BD inFlux® Cell Sorter
Users Guide
DRAFT

BD

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FCC Information

WARNING: Changes or modifications to this unit not expressly approved by the party responsible for compliance could void the user's authority to operate the equipment.

NOTICE: This equipment has been tested and found to comply with the limits for a Class A digital device, pursuant to Part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment. This equipment generates, uses, and can radiate radio frequency energy and, if not installed and used in accordance with the instruction manual, may cause harmful interference to radio communications. Operation of this equipment in a residential area is likely to cause harmful interference in which case the user will be required to correct the interference at his or her own expense. Shielded cables must be used with this unit to ensure compliance with the Class A FCC limits. This Class A digital apparatus meets all requirements of the Canadian Interference-Causing Equipment Regulations. Cet appareil numérique de la classe A respecte toutes les exigences du Réglement sur le matériel brouilleur du Canada.

Declaration of Conformity

This instrument and software conforms to the following council directives:

- 89/336/EEC (EMC Directive)
- 73/23/EEC (Low Voltage Directive)
- 98/37/EC (Machinery Directive)

This declaration of conformity is based upon the compliance of the product with the following harmonized standards:

- EN 61326-1 emission
- EN 61000   immunity
- EN 61010-1:2001
- EN 60825-1:1994 + A1:2002 + A2:2001

Revision History

Version	Date	Changes
2.4.1	May 2007	• Safety Label section • Operating environment
2.4.2	May 2007	• Updated safety info based on CE Cert. – in progress • Reformat & Update photos
2.4.3	August 2007	• Revise Declaration of Conformity • Update references to optical interlock with magnetic system
2.4.4	March 2008	• Revise operating specifications • Update selected photographs
2.4.5	April 2008	• Revise formats

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Preface

About this Guide

This manual is a resource for instrument operators, and is intended as an introduction to the instrument and its basic operation for new operators, as well as a reference for continued use by experienced operators.

The manual contains a basic description of the instrument, operating procedures, Spigot interface reference, and maintenance information.

The separate *BD inFlux Cell Sorter Safety Guide* includes all safety and hazard information.

Using this Guide

Operators of instrument are advised to read through this guide in its entirety prior to using the instrument. For maximum benefit, operators should be familiar with the various conventions used in this guide (e.g. safety cautions and text styles), which are outlined later in this chapter.

A familiarity with basic flow Cytometry concepts is assumed throughout the guide. For background on this subject, the reader is advised to consult a reference text such as *Practical Flow Cytometry* by Howard Shapiro.

Each chapter begins with a brief summary of the information contained in the chapter, and then presents information from general to specific.

Document Conventions

Alert Statements

This document uses the following conventions for alerting instrument operators to critical safety and other important information:

	DANGER
⚠️	**Refers to a hazard that, if not avoided, will result in death or serious injury.**

	WARNING
⚠️	**Refers to a hazard that, if not avoided, might result in death or serious injury.**

	CAUTION
⚠️	**Refers to a hazard that, if not avoided, might result in minor or moderate injury or in damage to the workstation.**

	NOTICE
⚠️	**Does not refer to a hazard, but is used to indicate important information.**

Instrument Description Conventions

This guide uses the following print type conventions when describing the instrument and Spigot:

Text Style	Meaning	Example
ALL CAPS	Instrument control, switch or knob	**PLATES**
ITALICS CAPS	Instrument display	*RECALL 1*
Italics	Emphasis	*Note:*
ALL CAPS SHADED	Spigot menu button or command	**FILE⇒PRINT LEFT**
Italics shaded	Spigot function button, drop down list or checkbox selection	*Sort!*

Getting Technical Assistance

Contacting technical support

If additional assistance is required, contact your local BD Biosciences technical support representative or supplier.

When contacting BD Biosciences, have the following information available:

- Product name, part number, and serial number
- Any error messages
- Details of recent system performance

Disclaimer

BD reserves the right to change its products and services at any time to incorporate the latest technological developments. This guide is subject to change without notice.

The information in this guide is provided to the customer as is, without any representation regarding its accuracy or completeness. Any reliance on or use of information contained herein is undertaken at the sole risk of the customer.

Limitations

This instrument is for Research Use Only. Not for use in diagnostic or therapeutic procedures.

BD Biosciences delivers software and workstations that are intended for running the instruments supplied by BD Biosciences. It is the responsibility of the buyer/ user to ensure that all added electronic files including software and transport media are virus free. If the workstation is used for Internet access or purposes other than those specified by BD Biosciences, it is the buyer/user's responsibility to install and maintain up-to-date virus protection software. BD Biosciences does not make any warranty with respect to the workstation remaining virus free after installation. BD Biosciences is not liable for any claims related to or resulting from the buyer/user's failure to install and maintain virus protection.

Note: The purchaser or lessee agrees not to use the instrument for sorting human sperm and that violations of this restriction will result in loss of any and all warranties associated with the instrument.

1

Introduction

Summary

This chapter provides an overview of this guide and the use of the guide in conjunction with instrument operation, and also describes conventions used in this guide.

Intended Use

	CAUTION
⚠️	**This instrument and software is intended for Research Use Only (RUO).**

The instrument is intended for use in laboratory research. Any investigational use of the device requires approval of appropriate authorities, such as the FDA. Approval for use beyond laboratory research is the responsibility of the study sponsor, not BD.

The instrument should only be operated by properly trained personnel in accordance with this guide and site-specific SOPs developed and approved by the study sponsor. All devices, such as lasers, used in conjunction with the instrument must be used as indicated by the original manufacturer.

Users are advised to read and understand the safety information in the Safety Guide that is included with this instrument.

Spigot Software

Spigot is a Windows-based software application that serves as the user's interface with the instrument. Spigot is the only software that should be used in conjunction with instrument. BD is not responsible for operation of the instrument with any software interface other than Spigot.

Running additional software applications while running Spigot is not recommended (with the exception of a data analysis package).

About Instrument Coordinate Conventions

The following coordinate systems are used in this guide to describe adjustment of the instrument.

Stage Adjustment Axes

The instrument employs adjustable platforms called "stages" for focusing and aiming both illumination laser light and light emitted as a result of fluorescence or scatter (discussed in more detail later in this guide).

Each stage is adjustable in the three Cartesian axes: x, y and z. The axes are defined with respect to either the illuminating light path or the path of emitted fluorescence or scatter, as follows:

X-Axis	**Moving the stage parallel to the light path (i.e. either to focus the illumination beam or focus the fluorescent/scattered light spot).**
Y-Axis	Sweeping the stage horizontally (and perpendicular to the light path).
Z-Axis	Moving the stage vertically (and perpendicular to the light path).

Nozzel Adjustment Angles

In addition to the three nozzle stage movements, the instrument nozzle assembly allows two further adjustments: Theta (Θ) and Phi (Φ) angles, defined (as viewed from the front of the instrument) as follows:

Θ	**Theta angle refers to a side to side rotation of the stream about the nozzle tip.**
Φ	Phi angle refers to an in/out rotation of the stream about the nozzle tip.

Well Deposition Unit Axes

With respect to movement of the Well Deposition Unit (WDU) collection tray, X-axis is a "right-left" movement, while Y-axis is an "in-out" movement.

Instrument Overview

Summary

This chapter provides the user with an overview of the instrument. First, a pictorial tour presents the instrument. The second half of this chapter introduces the functional subsystems, and provides a brief description of each subsystem.

Functional Description

The BD inFlux high speed cell sorter is a research instrument that brings several technologies together to provide high-speed detection, analysis and sorting of particles excited by laser light. Each one of these technologies, discussed in further detail below as a functional subsystem, is critical to successful cell sorting.

The instrument focuses laser light on a fast-moving, thin stream of particles (e.g. cells, chromosomes, organisms) and observes the fluorescence and light scatter emitted by these particles using sensitive photo multiplier tubes (PMTs). High-speed electronics capture these data, and depending on user-defined parameters, sort/no sort decisions are made almost instantly.

The instrument nozzle causes the stream to break into droplets in a predictable fashion. The instrument selectively places an electric charge on individual droplets containing desired particles to be sorted. High voltage plates then deflect the individual droplets from the stream for separate collection.

Operating Environment

Mains voltage (North American Models):	115-120VAC/60Hz ,8A (Cytometer), 5A (Optional HEPA enclosure)
Mains voltage (European Models):	230VAC/50Hz, 4.5A (Cytometer), 2.8A (Optional HEPA enclosure)
Temperature:	18 °C – 25 °C
Humidity:	5%-80% non-condensing
Air Pressure:	90 PSI / 6.2 Bar Max,0.2l/min @ 60PSI
Vacuum:	2.5psi / 0.17 Bar or greater, 50l/min recommended

Pictorial Tour

The following photographs provide a brief tour of the BD inFlux and introduce the operator to subsystem and component terminology.
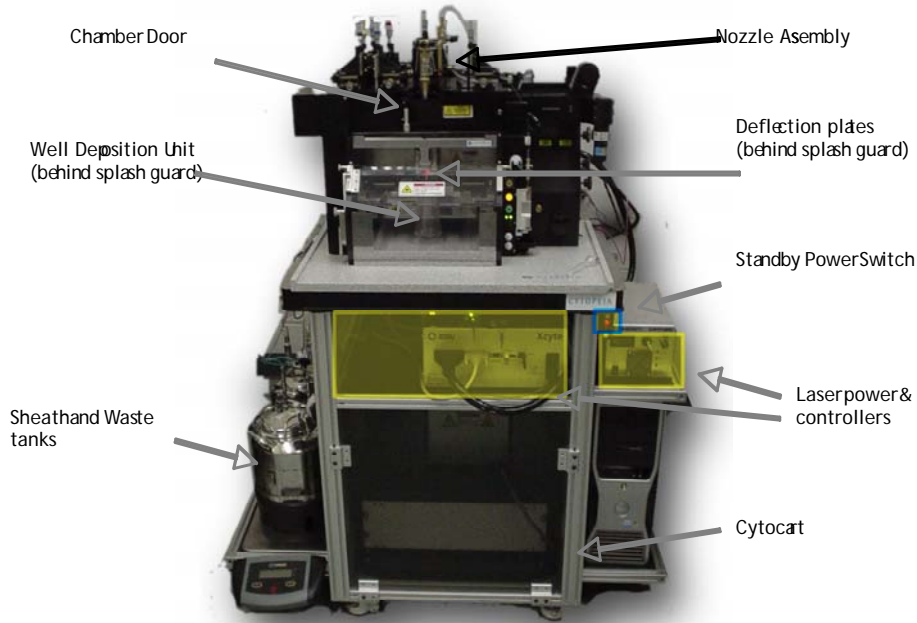
Figure 2.1: BD inFlux instrument front view
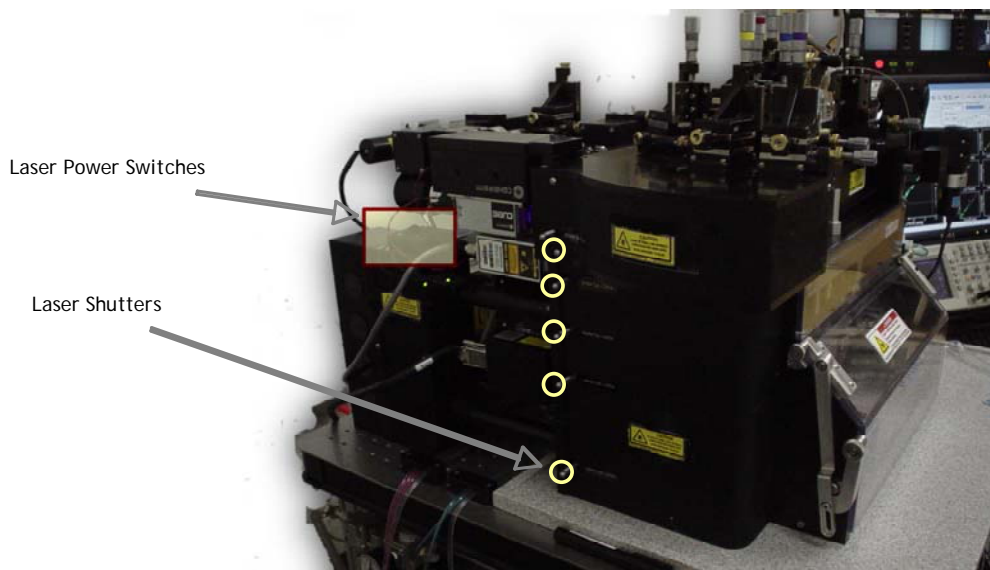
Figure 2.2: BD inFlux sort head left side

Illumination Steering Optics

Image Block

Detector Modules

Laser

Pressure Console

Drop Camera Micrometer

Laser Stages

Nozzle Stage

Forward Scatter Detector Stage
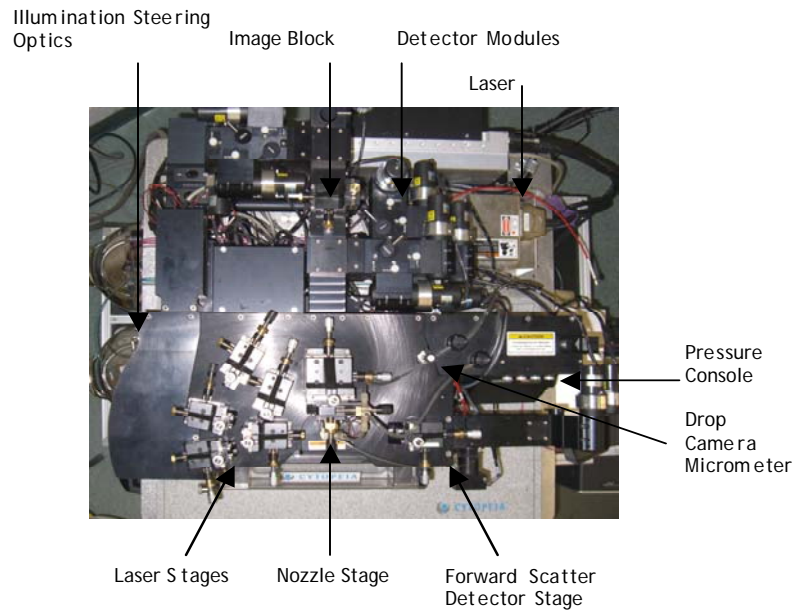
Figure 2.3: BD inFlux sort head overhead view

Sample Valve

Bubble Detector

Pressure Regulator controls

Fault LEDs

Sample Station

Sample Cooling accessory ports

Boost Button

Sample offset

Main Air Switch
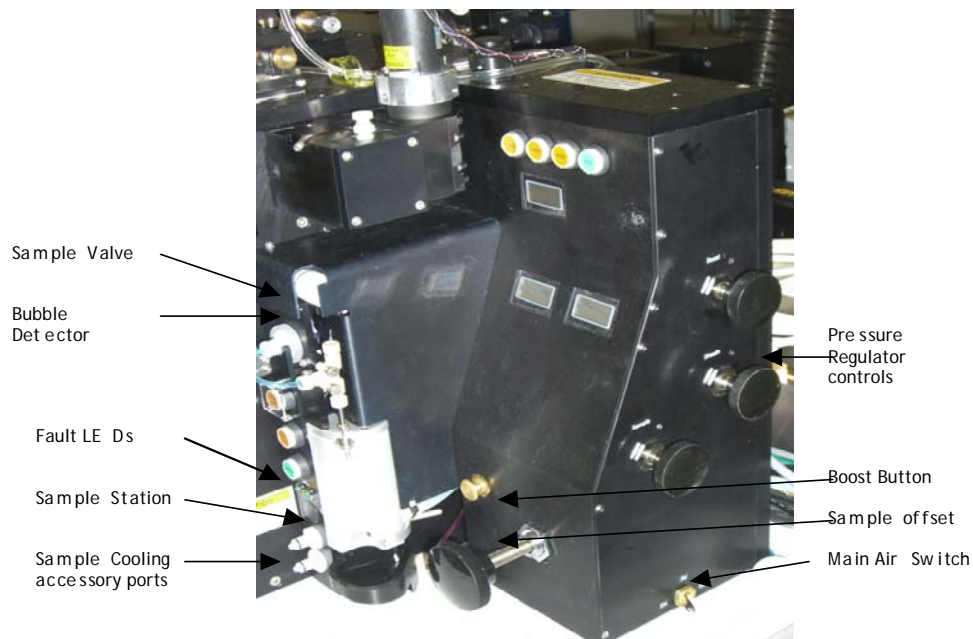
Figure 2.4: Sample control box and pressure controls
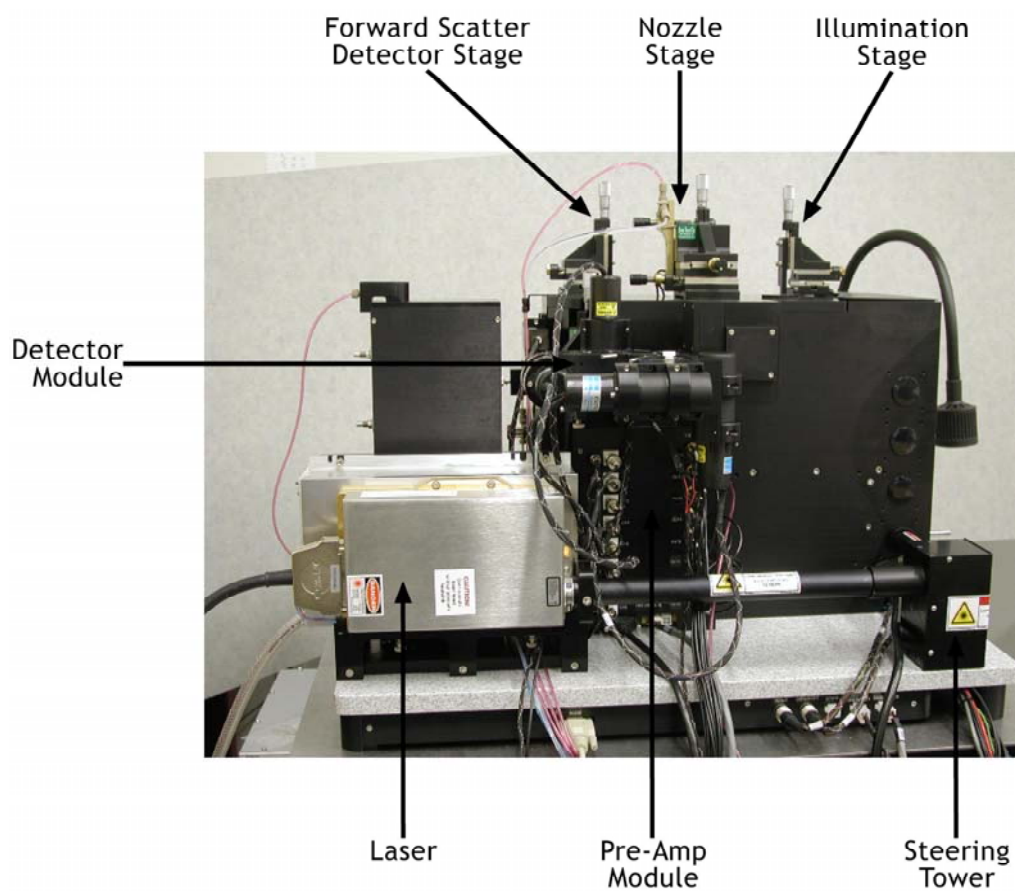
Figure 2.5: BD inFlux sort head rear view
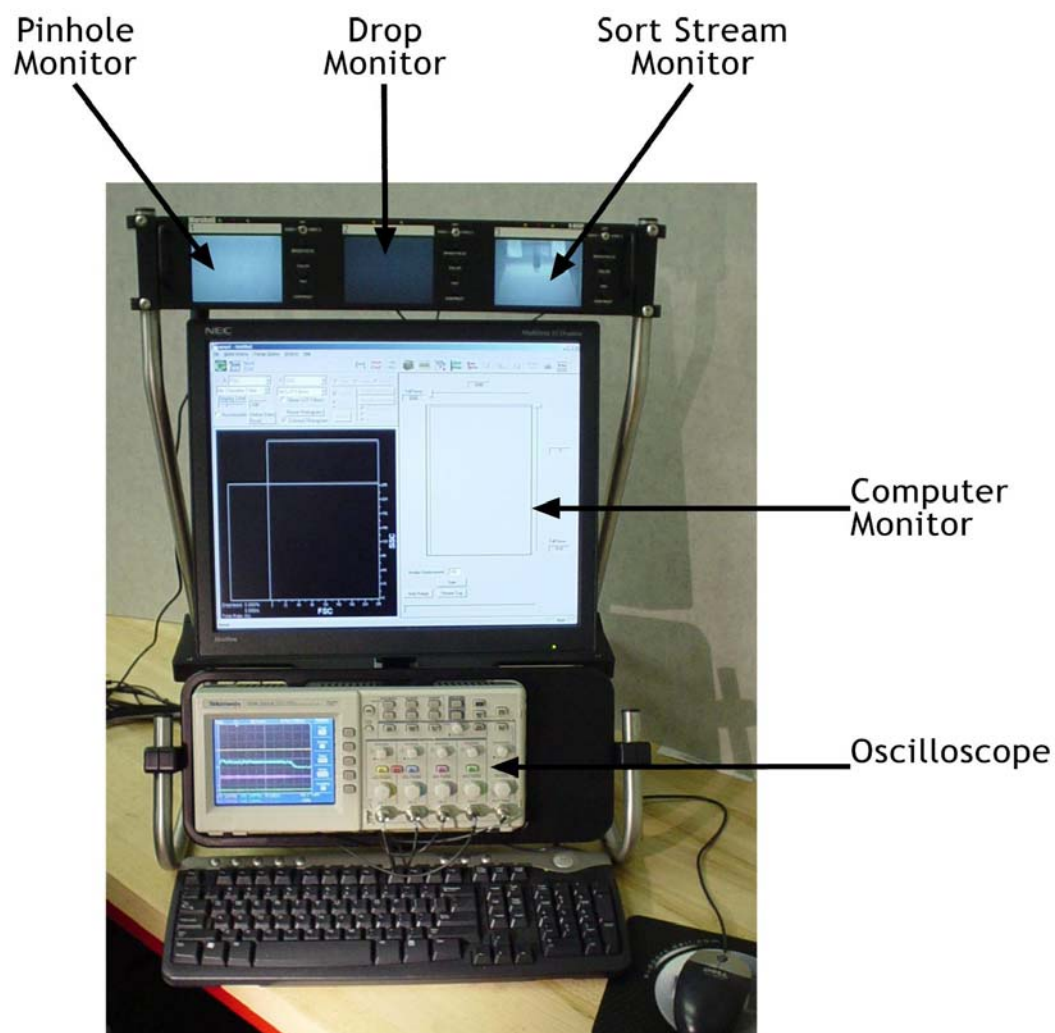
Figure 2.6: Sort rack controls
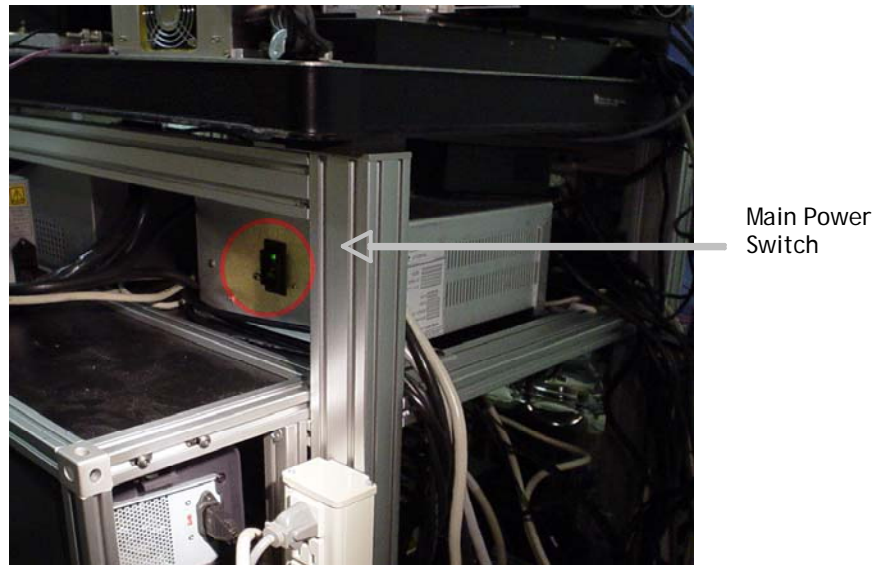
Main Power
Switch

Figure 2.7: Location of main power switch

Functional Subsystems

This section briefly describes the primary functional subsystems of the instrument and lists the main components of each subsystem.

Fluidics

The fluidics subsystem includes the components necessary for sample introduction, sheath fluid management, pressure regulation and monitoring, and waste fluid recovery.

Sample introduction

This involves placing a stream of sample particles, typically suspended in saline, in the center of a larger stream of sheath fluid (saline). Then, under carefully regulated pressures, this stream is forced through a tiny orifice in a nozzle, producing a fine, fast-moving jet of fluid, with sample particles in the center.

The main components of sample introduction are the sample station, the sample line, and the nozzle assembly

Figure 2.8: Sample Station

The sample station includes a tube holder for loading sample tubes, a sample valve, an air detector and buttons for controlling the sample valve marked **SAMPLE**, **BACKFLUSH**, and **OVERRIDE**.

The sample line delivers the sample from the sample station to the nozzle assembly. The sample line is made from PEEK tubing and has an inner diameter of 254 micrometers. It is designed to minimize sample carryover and is easily replaceable.

Sample is delivered to the nozzle assembly, where it enters the center of a stream of sheath fluid. The nozzle assembly also includes a piezoelectric element used to force the stream to form droplets

Sheath fluid

This is so named because the sample fluid is surrounded by a sheath of saline as it is forced through the nozzle, causing the sample to be centered and lined up in the jet emitted from the nozzle tip.

The main components of sheath fluid management are the Sheath Reservoir, a digital scale for monitoring the level of fluid in the Sheath Reservoir, the sheath line and sheath valve, and the nozzle assembly.

Pressure regulation

This subsystem applies an adjustable level of air pressure to the sample tube and to the Sheath Reservoir. It also involves a means for monitoring the sample and sheath pressures.

The main components of pressure regulation are the pressure console and the several pressure lines emanating from it.

Valve mode

Valve mode for the sheath and purge valves is controlled by buttons at the top of the pressure console. The buttons toggle their respective modes on and off.

Available modes are: **RUN**, **RINSE**, **PURGE**, and **OFF**. The **PULSE** button initiates a short burst of debubbling pulses.

Note that when the run mode is enabled, the other buttons are locked out. **RUN** must be turned off before another mode can be enabled.

Figure 2.9: valve mode controls

Waste fluid recovery

This involves retrieving all fluids that have been run through the instrument, except those that have been separately collected during the sort process.

The main components of waste fluid recovery are the waste drains and buckets, the purge line and valve, the waste lines, the Waste Reservoir and the vacuum pump.

Illumination

The illumination subsystem includes all the components necessary to apply a focused beam of laser-emitted light to the jet exiting the nozzle tip.

The main components of the illumination subsystem are:

- Laser(s)
- Protective shields and guards
- Laser shutters and the shutter interlock system
- Alignment prisms and/or mirrors
- Iris diaphragms
- Focusing lenses mounted on adjusting stages.

The instrument is equipped with embedded lasers mounted in two areas: on the optical bench behind the main sort head, or attached directly to the rear of the sort head.The laser beams are deflected into the sort head as illustrated below:
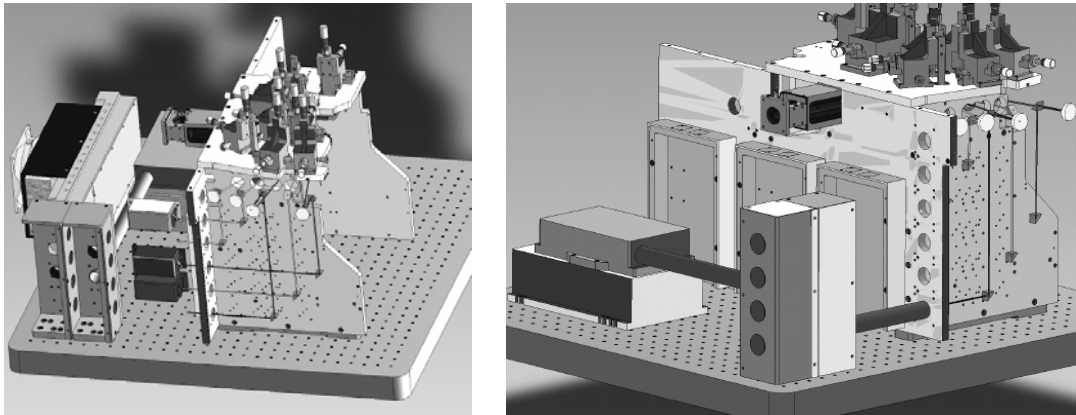
Figure 2.10: Laser beam paths on the BD inFlux sort head: side-mounted lasers (left) and rear-mounted lasers (right)

Detection

The instrument detection subsystem is modular and open in its design. Its purpose is to spectrally separate and quantify the intensity of light emitted from the particle stream as a result of fluorescence when excited by laser radiation.

The detection subsystem also includes the components necessary for light scatter intensity measurement.

The main components of the detection subsystem are:

- Image block assembly
  The image block assembly contains the objective lens, the pinhole mirror and prisms for directing the light into the detector modules.
- Detector modules.
  The detector modules contain band-pass filters, dichroic mirrors and photo multiplier tubes (PMTs).

Signal Processing

Signal processing encompasses all of the electronics components required to control PMTs and amplify and process PMT signals into data that can be monitored by the operator, retrieved and analyzed by Spigot and used by the sort electronics subsystem.

The main components of signal processing are:

- Logarithmic and linear pre-amplifiers (log and lin preamps)
- Analog to digital converters (ADCs)

Sort Electronics

The sort electronics subsystem contains the electronics components necessary for sorting particles. This process includes transferring sort gating information from Spigot into the hardware look-up tables using data provided from signal processing to make sort/no sort decisions based on the inputted criteria, and controlling the electrical components involved in the formation, tracking and sorting of individual droplets.

The main components of sort electronics include:

- Circuit boards for hardware look-up tables
- Counters
- Piezo drive
- Stream deflection
- High voltage deflection plates
- Control circuitry

Many of these components are housed in the Electronics Console.

Sample Collection

After droplets are sorted, the sample collection subsystem is responsible for collecting those droplets. The instrument enables operators to sort into standard test tubes, a 96-well tray, or onto standard microscope slides.

The main component of sample collection is the Well Deposition Unit (WDU), which consists of a two-axis moveable tray powered by stepper motors and controlled by the operator via the Spigot interface.

The tray can hold either a 96-well tray or the tray insert, which holds either tubes or slides.

Users may program the WDU to collect in any pattern within its range (for example 384-well tray, 50ml tube, etc.).

Monitoring

The monitoring subsystem enables the operator to observe, in real-time, data acquired by the instrument, to see feedback on system settings, and to view close-up images necessary for proper adjustment and monitoring of the instrument.

The main components of the monitoring subsystem are:

- Spigot software interface
- Oscilloscope
- Triple LCD camera monitors: pinhole, drop, and sort stream

Other monitoring components are:

- Pressure readouts
- Sample station fault LED indicators
- HV plate readouts
- Drop position indicator

Power Distribution

The power distribution controls enable the operator to turn on specific parts of the instrument as needed. Power is distributed to the instrument components through a number of switched and unswitched power strips.

Power to the instrument subsystems is typically controlled through the standby power switch, and through the individual power switches associated with each laser.

These switches are located on the controllers at the front of the instrument or at the left side of the instrument mounted adjacent to the laser heads.

The main power switch is located on the isolation transformer at the rear of the instrument (Illustrated in Figure 2.7).

In normal operation, the main power switch is to remain on. However, should it be necessary to power down all subsystems, the main power switch may be used.

3
Operation Tutorial

Summary

This section contains detailed step-by-step instructions for operators new to the instrument. The steps will guide the user through set-up of the instrument and the completion of a simple sort protocol.

Information on the use of the Spigot software application is presented as part of these instructions; however users may also refer to the Spigot interface chapter for a more detailed explanation of Spigot software.

⚠️	**Warning**
	Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure.

Preparation

Collect supplies

Gather all supplies necessary for instrument set-up.

These typically include:

- Sheath and waste reservoirs
- Nozzle tip
- Nozzle back flush reservoir
- Sample tubes
- Cotton swabs
- Paper towels
- 10ml syringe
- Microscope slides.

Make sure that all supplies are clean.

Prepare fluidics reservoirs

Fill the sheath reservoir with up to 7 liters of clean sheath fluid filtered to 0.2 microns.

Ensure that the waste reservoir is clean and empty.

Attach lids and quick connect fittings from instrument fluidics lines to the reservoirs.

Note that each reservoir port is unique, so that each fitting connects to only one port.

Power-up

During normal use and storage, the main power switch remains in the on position, providing standby power to subsystems as needed.

Subsystems are powered on individually following the steps below.

Start Computer

Turn on the computer by pressing the power button on the front of the case.

⚠️	**Note** **It is important that the computer be started before the Electronics Console.**

Power-up Electronics Console

Turn on the Electronics Console by flipping on the auxiliary power switch, shown in Figure 2.1. The switch will be illuminated when power is on.

When the auxiliary power is switched on, the following peripheral devices will be turned on:

- Oscilloscope
- Laser shutters
- All sort electronics
- Video cameras and monitors

Start Spigot

Wait at least 10 seconds after powering up the electronics console before starting Spigot .

Launch Spigot either from a desktop shortcut or from the start menu.

Start Lasers

Most lasers require at least 10 minutes of warm-up time. Close all laser shutters on the instrument and start all lasers in accordance with the manufacturers' procedures.

Typically this entails powering on the lasers and then turning the associated key switch to the enable position. Key switches must be explicitly enabled after powering on the lasers. Laser control units and their locations are shown in Figure 2.1 and

Figure 2.2 above. Some laser power controllers have settings to regulate the power output.

Power up Fluidics

Start fluids early. Fluids take from 30 minutes to one hour to fully stabilize. It is therefore important to start the fluidics as early as possible.

Apply Vacuum

Turn on the vacuum pump or other vacuum supply.

Read the gauge of the waste reservoir and ensure that it reads at least 5" Hg. If less than 5" Hg is observed after the tank has a few moments to come to pressure, tighten the lid to ensure that a seal is formed.

It is necessary for the waste reservoir lid to be tightened as much as possible without using a wrench.
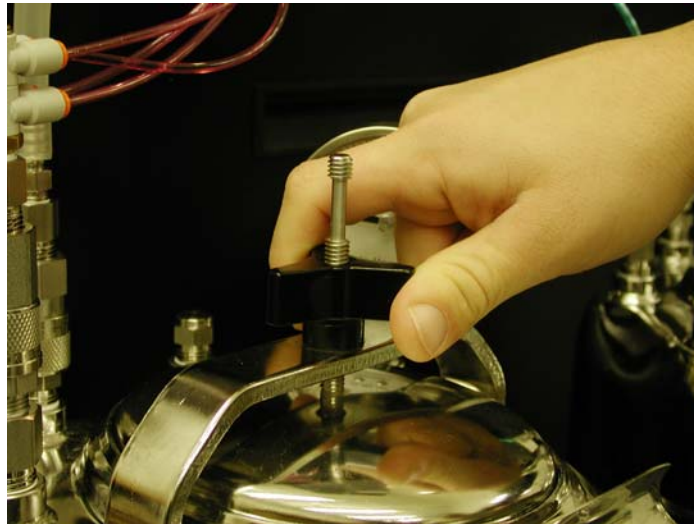
Figure 3.1: Tighten Waste Reservoir lid

Set Valve Mode to OFF

Make sure that the valve mode is set to off (no mode buttons on pressure console are illuminated.

Choose Pressure and Frequency Settings

The instrument is capable of running with several different nozzle tip sizes, each of which has many different pressure/frequency combinations.

The instrument droplet rate (frequency) is set by Spigot and ultimately determines the practical upper limit for running particles when sorting.

A good rule of thumb is to have a *droplet rate that is about ten times the desired sample rate*, if possible, in order to minimize coincident (two particles in one droplet) events.

After choosing a pressure/frequency setting pressurize the reservoir and set the sheath pressure following the procedure below.

The droplet formation is discussed in detail in later sections. For this section it is important only to set the desired sheath pressure.

See Appendix A for a chart of available pressure/ frequency settings.

Pressurize Sheath Reservoir

Close the pressure relief valve on the sheath reservoir. Turn on air supply by flipping up the **AIR** switch on the Pressure Console.

Observe the gauge on the sheath reservoir to ensure that it is being pressurized (below left). Reseat the lid of the sheath reservoir if the pressure in the reservoir does not increase.
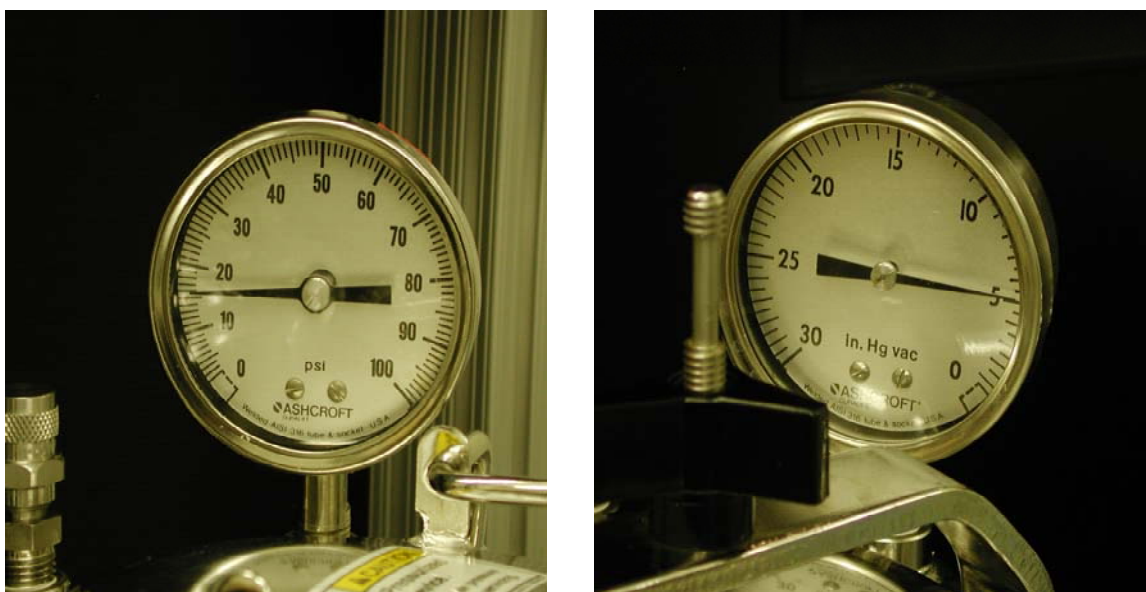
Figure 3.2: Sheath and Waste Reservoir gauges

Set the Sheath and Sample Pressures

The pressure console includes four regulators:

- Sheath
- Sample
- Sample Offset
- Boost

Both the sheath and sample regulators can regulate an input pressure (of at least 20 PSI over the desired setting) to 3-100 PSI, although operating pressure should generally be kept below 80 PSI.

The Sample Offset regulator adds 0-5 PSI to the sample regulator, allowing fine control of the sample rate.

The boost regulator is set to add 3 PSI to the sample pressure when the boost button is depressed and should not usually be adjusted.

Use the regulator knob labeled *SHEATH* on the right side of the pressure console to set the sheath pressure to the desired value.

Note that it may take some time for the reservoir to stabilize to a constant pressure setting.

To balance the sample pressure with the sheath, follow these steps:

1. Dial out (decrease) the regulator knob labeled **SAMPLE OFFSET** on the front of the pressure console until it no longer lowers the sample pressure readout on the pressure console.

2. Use the regulator knob labeled **SAMPLE** on the side of the pressure console to adjust the sample pressure until the *SAMPLE PRESSURE READOUT* is 1 PSI lower than the sheath pressure readout.

3. Use the **SAMPLE OFFSET** regulator knob to increase the sample pressure readout to 1 PSI greater than the sheath pressure.

This process ensures that the sample offset regulator is adding about 2 PSI to the total sample pressure, and also leaves the sample to sheath differential pressure at about 1 PSI – typically a good starting point when running particles.

Stream Generation

Flush system
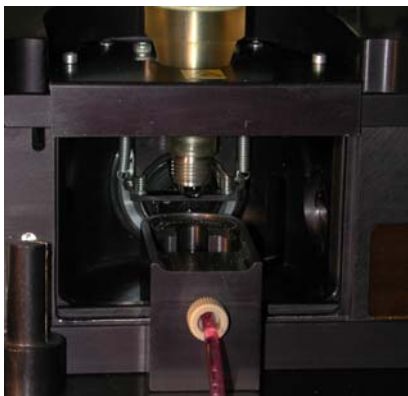
Figure 3.3: Flush bucket in place under nozzle with nozzle tip removed

With the nozzle tip removed, place the flush bucket under the nozzle. Recheck that the waste reservoir has an adequate level of vacuum applied to it.

Press the **RINSE** button to open the sheath and purge lines. Take care not to overfill the flush bucket, if necessary reduce sheath pressure to 15-20 PSI to prevent overfilling.

Run fluid through the lines for at least 30 seconds, ensuring the lines are mostly full of fluid. Press the **RINSE** button to close the sheath and purge valves and stop the flow.

Clean and attach nozzle tip

Initially clean the nozzle tip by sonicating it for approximately 5 minutes. Then use a syringe to further clean by flushing 0.2 μm filtered water (de-ionized, distilled, or other), sheath fluid, or mild detergent through the nozzle tip in two steps as shown below, flushing "backward" through the tip first.

Take care not to let any contaminants enter the nozzle tip.
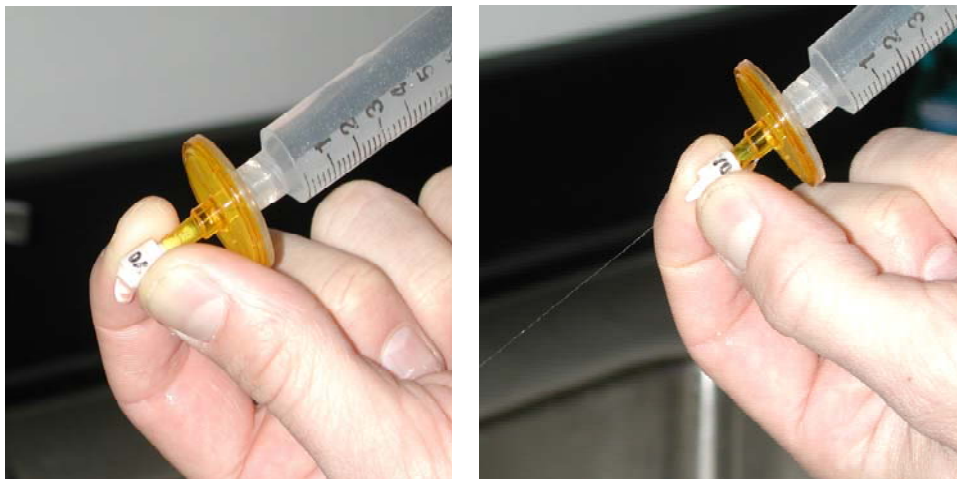
Figure 3.4: Clean nozzle tip with syringe from both directions

Install the nozzle tip by carefully placing the nozzle tip in the nozzle nut (large tweezers can be used to avoid contamination).

Be sure that there is an o-ring around the nozzle tip inside the nozzle nut. Screw the nozzle nut onto the nozzle and tighten as much as possible *without using a wrench*.

⚠️	**Warning**
	The nozzle and stream are charged by the drop charging circuit. Ensure that spigot is running and the test deflections are off before attaching the nozzle.
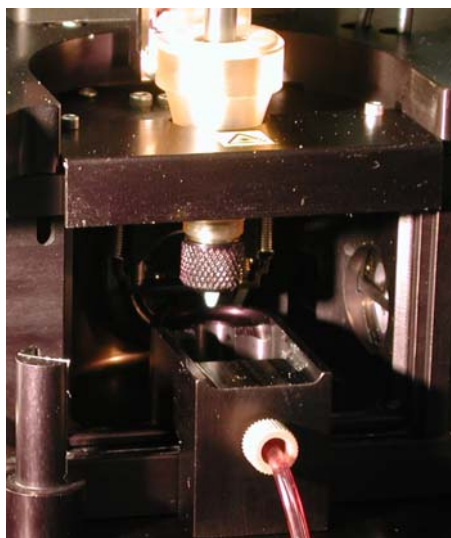
Figure 3.5: Nozzle tip and nut installed

Start a stream

Press **RUN** to open the sheath valve and start a stream.

Be sure to return the sheath pressure to the desired operating pressure if adjusted during rinsing.

Back flush and de-bubble

Make sure that a sample tube is not loaded and that the sample tube lever is in the open position.

Back flush the sample line by pressing **BACKFLUSH** on the sample station.

Allow the sample line to drip sheath fluid for about 30 seconds. Press **BACKFLUSH** again to close the sample valve and complete back flushing.

Press **RUN** to deactivate the run mode, which will close all valves.

Purge the nozzle of bubbles by placing the clean nozzle purge reservoir filled with sheath fluid atop the flush bucket and pulling that fluid up through the nozzle.

Figure 3.6: Nozzle purge reservoir rests in place on flush bucket
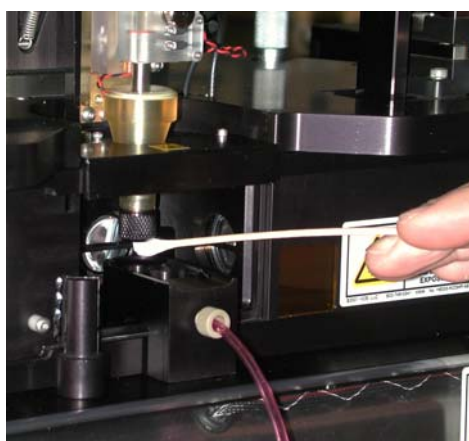
Figure 3.7: Swab excess fluid from sides of nozzle tip

Ensure that the nozzle tip is submerged into filtered sheath fluid in the nozzle purge reservoir.

Press the **PURGE** button to open the purge valve and pull fluid up through the nozzle tip and purge the system of air. Bubbles should be observed as they are pulled up through the nozzle.

When all bubbles have traveled past the purge valve press the **PULSE** button to free any other bubbles lodged in the nozzle.

Allow freed bubbles to travel past the purge valve and **PULSE** again.

Continue pulsing and purging until no sizable bubbles are freed from the nozzle. If necessary, add sheath fluid to the nozzle back flush reservoir (a syringe with filter works well) to keep the nozzle tip submerged throughout this process.

Push **RUN** to start a stream before removing the reservoir.

Remove the back flush reservoir and use a cotton swab or similar to remove excess fluid from the outside of the nozzle tip.

Ensure that the stream is emitted straight out of the nozzle tip. If the stream is even slightly askew or wanders, return to the top of this subsection and repeat cleaning the nozzle and flushing the system.

Align stream

Make sure that an Erlenmeyer flask is placed under the stream drain. For most instruments this is accomplished by putting the Erlenmeyer flask in the WDU and setting the WDU position to **SAFE**. In the _Sort_ screen of Spigot .

Remove the flush bucket so that the stream passes through the illumination chamber into the sort chamber.

Press the **ILLUM** button on the Monitor Stand to turn on illumination of the stream near the drain.

The stream may or may not land in the stream drain, but should be captured by the Erlenmeyer flask if it misses the stream drain. If not, take measures necessary to prevent spills.

Alignment of the stream consists of two steps: bringing the stream into the focal point of the microscope objective and ensuring that the stream is properly positioned.

Use the pinhole monitor and the sort stream monitor for primary visual feedback while making adjustments to the nozzle stage.

Bring stream into focus

Use the x-axis (focus), y-axis (left-right) and z-axis (up-down) micrometers of the nozzle stage to focus and position the stream in the pinhole monitor as shown below.

The stream should be centered on the pinholes, sharply focused, and the nozzle tip should be barely visible in the top of the monitor.

When performing this procedure, it may be helpful to use the provided LED torch to illuminate the stream. The illuminated stream should appear as in Figure 3-8.

Position stream

Once the stream is properly focused in the pinhole monitor, use the Θ-angle and Φ-angle micrometers of the nozzle stage to aim the stream at the drain in the sort stream monitor.

Iterate several times between the above steps until the stream is focused and positioned in the pinhole monitor and also aimed at the drain in the sort stream monitor.

Figure 3.8: Stream focused on pinhole mirror (left) and stream aimed at drain (right)

⚠	**NOTICE** **The stream may splash and spray out of the drain if running at high pressures (over 30 PSI) and droplets are not being generated. See section 4.11 for information on droplet generation.**

Illumination

Illumination consists of steering the laser paths so that they intersect the stream at the proper locations. The following steps should be repeated for each illumination laser.

Open laser shutter

Figure 3.9: Manual laser shutter, in open position

Close the chamber door slide and put the chamber lid in place. Open the manual laser shutter on the left side of the sort head.

The instrument is also equipped with an automatic laser shutter interlock system. When the system is armed, opening the chamber door slide will cause the automatic shutter to close.

It will not reopen unless two conditions are met:

1. The chamber door slide is closed, and
2. The system is reset by placing a finger in front of the reset detector.

The reset detector is behind a small hole in the upper right corner of the chamber door, as shown below.
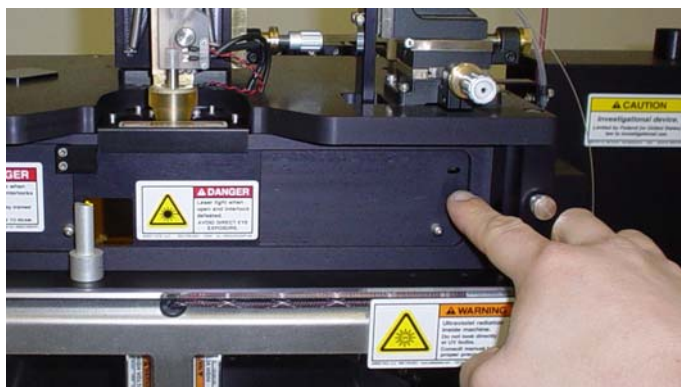
Figure 3.10: Laser interlock system reset aperture

Steer laser to stream

Note: Most lasers will show a glow in the pinhole monitor when they strike the stream. All lasers will scatter off of the stream.

Visible lasers will create a horizontal scatter line across the scatter bar as observed through the opening around the nozzle (Figure 3.11).

This procedure can be more challenging with UV lasers due to their invisibility. Fine-tuning of the laser will be completed later, when running calibration particles.

Lasers will usually be very close to alignment from day to day and this step may not be necessary.

Use the illumination stage's y-axis (sweep) and z-axis to aim laser at both the center of the stream and at the height of the appropriate pinhole. The laser will not need to be focused except for special applications.

Repeat this process for all laser paths.
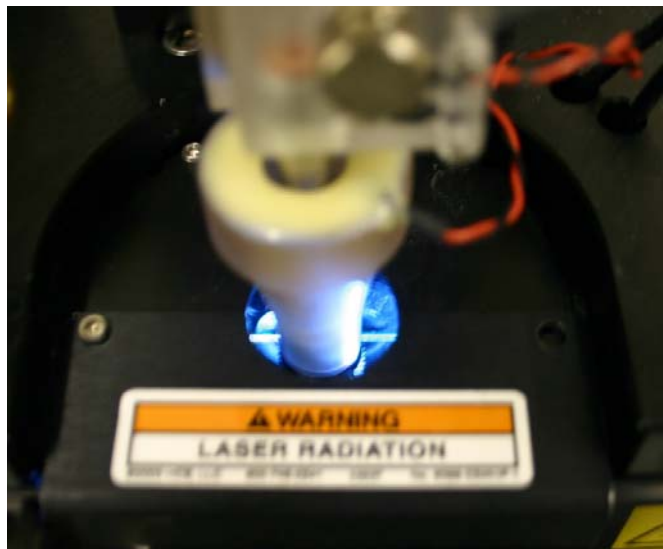
Figure 3.11: scatter line appears inside the chamber when laser is aligned.

Sample Introduction

Load sample tube

	NOTICE
⚠️	**Laser alignment requires fluorescent calibration particles. BD recommends beads with broad excitation and emission spectra such as Spherotech Ultra Rainbow particles #URFP-30-2.**

Use only Falcon 35-2063 5 mL polypropylene sample tubes. BD recommends that all samples be filtered to 40 μm, or about half of the nozzle tip size, to prevent nozzle clogs and ensure optimal sorting.

Make sure that the Fault LEDs are both lit.

- If the Left LED is not lit, then there is fluid in the air supply line – close the Sample Lever without a tube loaded to blow any fluid out.
- If the right LED is not lit, then there is air in the sample line – open the sample lever and press **BACKFLUSH** to purge the sample line of air.

Fill sample tube with up to 3 mL of sample.

Load sample tube into sample tube holder and lock tube in place over the stopper using the lever. Make sure that the sample lever is completely closed.

Take care not to damage the sample tube.

Figure 3.12: Locking sample tube in place

	CAUTION
⚠️	**Use care when removing the sample tube; the sample tube is usually pressurized and may spill or splatter sample fluid.**
	To properly remove the sample tube, move the lever slowly backward until the tube is pushed down into the recessed area of the lever. Pressure is then released and the tube may be removed safely.

Open SAMPLE VALVE

Check that the sample pressure is set at about 1 PSI over the sheath pressure.

Check that both small green LEDs are lit on the front of the fluidics station, if not backflush the sample line and/or close the sample lever without a sample tube loaded until both are lit.

Press **SAMPLE** to open sample valve and begin running sample. **BOOST** may be depressed for a few seconds quicken sample delivery to the nozzle tip.

Run sample at a low rate

Aim the laser so that it is just above the center pinhole, in-between pinholes for multi-laser systems.

When running bright calibration beads or cells at a low flow rate a narrow sample core can be viewed in the pinhole monitor.

- If the flow rate is too high, the sample core will be very large, approaching the size of the jet.
- If no beads are observed flashing in the stream then the flow rate is too low.

Figure3.13: Pinhole camera image, with beads showing in stream

The flow rate of the sample is determined by the sample pressure setting. At sample pressures less than1 PSI over sheath pressure a low flow rate is usually achieved, however this setting depends on the concentration of the sample.

When first running a sample you may wish to boost the sample pressure temporarily in order to get sample to the jet quickly. Hold down the boost button to boost the sample to 3-5 PSI over the sheath.

Adjust the sample pressure until a low flow rate is achieved.

Adjust the y-axis of the illumination stage so that the brightest signal is observed in the pinhole monitor.

Adjust the x-axis of the nozzle stage so that the signal is tightest (spatially) in the pinhole monitor.

Iterate between these steps until the sample core is as narrow and bright as possible. This step insures that the microscope objective is focused on the sample core and that the laser is aimed at the proper point.

Use the z and y-axes of the nozzle stage to place the sample core in the center of the pinhole.

The sample valve may be closed by pressing **SAMPLE** if desired at this point to preserve the sample until data acquisition parameters are set up.

Data Acquisition

Spigot software is used for most all data acquisition controls. Spigot allows control of PMTs and allows custom gating schemes to be developed and saved in configuration files.

It is recommended that a configuration file be saved for each protocol run on the instrument.

See the chapter on Spigot interface for detailed descriptions of Spigot functions.

This chapter will refer to fluorescence channels as FL1-FL7 and forward and side scatter as FSC and SSC.

Open a Spigot Configuration File (*.sco)

If a Spigot configuration file has already been saved for alignment particles, open that file and skip the steps outlined below for configuring Spigot . Otherwise, follow the directions below to set up a new configuration and save the settings in a new *.sco file by selecting **FILE⇒SAVE CONFIGURATION FILE** in Spigot .

Activate PMTs

In the **SIMPLE CONFIG** pane, activate the main PMT power and all PMTs that will be used during alignment.

It is crucial that the FSC PMT be activate since it is the signal that is (typically) used for the system trigger. Thus, no data will be acquired unless the FSC PMT is activated.

Choose parameters to plot

Choose parameters to plot in the Spigot left display as described in the Spigot Interface chapter.

Typically, FL1 is plotted vs. FSC and these instructions will detail how to set-up and align to those two parameters.

Use a similar method for plotting other or more parameters.

Set initial PMT gains

The instrument is usually shipped pre-configured for linear (lin) signal amplification for scatter channels and logarithmic (log) amplification for fluorescent channels.

These settings can be changed in from Spigot in the **SIMPLE CONFIG** menu, see chapter 5 for more details.

Activate PMT 1 and PMT 3 (FSC and FL1) by clicking on the PMT power buttons for those channels. Notice that the channel number will turn from red to green, indicating that the PMT is powered on.

In the left display, make sure to plot FSC vs FL1. PMT gains may be adjusted either by using the slider in the **SIMPLE CONFIG** menu, or by using the small orange triangle sliders in the left display.

Note that the keyboard arrows may be used to fine-tune the gain after sliding or clicking on the slider.

Use one of the methods described above to increase the FSC gain until pulses of about 5 V can be detected on the oscilloscope. Do the same for FL1.

Since the range for pulse height measurements in Spigot is 0-10 V, pulse heights of about 5 V should correspond to data that is acquired in about the middle of a FL1 vs. FSC plot in the left display.

Make sure that data are being acquired by Spigot and adjust that PMT gains so that the data are not off-scale.

Set Trigger Level and Event Trigger Delay

Trigger Level sets the threshold for the lowest detectable signal and it set off of the system trigger (usually FSC). Its intended use is to eliminate noise.

It is usually best to keep the _Trigger Level_ as low as possible since the system will not detect any events that fall below the threshold, and those events may end up in the sorted sample.

With multi-laser systems it is necessary to set the _Event Delay_ for each additional laser.

Align additional lasers by first using the pinhole monitor to aim the laser at the appropriate pin hole and then fine tune the laser looking at the pulses on the oscilloscope.

Adjust the _Event Delay_ from the menu until the signals from additional lasers fall into

appropriate "bins" as displayed on third oscilloscope trace (labeled as the ALL cable).

Finally make sure that additional laser channels are set to the appropriate events (1, 2 or 3) in the SIMPLE CONFIG screen.

Typically, *Event Delay* will only need to be changed if the sheath pressure of the system is changed.

Alignment

Fine-tuning of the instrument involves positioning the sample core at the focal point of the objective lens in the pinhole, illuminating the sample core optimally, and aligning the FSC. PMT gains may need to be adjusted as the system is tuned so as to keep the data on scale.

Focus light emission from sample in stream

Open sample valve and ensure that sample is flowing at a low rate. Ensure that the sample core is focused, maximally illuminated, and in the center of the pinhole using the pinhole monitor, nozzle stage and illumination stage as described earlier in this chapter.

With beads running, it is possible to focus the stream so that the sample core is the focal point for the objective lens.

First make a small adjustment to the x-axis of the nozzle stage.

Next, use the y-axis of the first laser illumination stage to sweep the beam across the stream until the bead signal is a bright as possible.

Repeat these steps until the sample core is as focused as possible (sample core looks like a tight line) and is as bright as possible.

Optimize a fluorescence channel

While a FSC signal must be detected first (if it is the system trigger), it is important to first align to a FL or SSC channel since the objective lens is fixed while the forward detector stage allows adjustment.

In the left pane, use the slider to set the *Display Limit* to 1, this reduces the number of historic events shown on screen, making adjustments more visible.

Some flow cytometer operators prefer to align the FL signals using linear amplification.

To do so, choose LIN for the amplification for the appropriate channel from Spigot SIMPLE CONFIG Menu.

Remember to set the channel back to LOG after tuning and use Spigot to re-adjust the PMT gain appropriately.

Adjust the y-axis of the illumination stage and use the oscilloscope pulse monitor and the left spigot screen to maximize the signal from the FL1 detector.

The pulse should be as high as possible and the signal in Spigot should be maximized.

Remember to ignore the FSC signal while tuning the FL channel.

Optimize forward scatter signal

Forward scatter does not generally need to be aligned day to day, although it can be aligned using the following procedure.

Once the FL signal is optimized, the FSC signal can be optimized.

Use the y- and z-axes of the forward scatter stage to maximize the signal from the forward scatter detector.

Optimize additional laser paths

After the first laser path is aligned for fluorescence and FSC, additional laser paths can be aligned for fluorescence using their respective illumination stages as described above.

Sort Gating

Sort gates can be defined via the Spigot interface. Up to 12 (six left and six right) 2-parameter sort windows can be defined at a time.

Sort windows are two-dimensional sort gates that allow the operator to select portions of data to be sorted. Many options, such as sort filtering, are available.

These instructions will describe how to set up a basic sort window as well as a few advanced features. The Spigot interface chapter also contains reference information.

Draw Sort Windows in Spigot software

To draw a sort window (SW) in Spigot software first click on the **SORT** menu. The right display will contain six squares corresponding to the instrument's six Look-Up Tables ("LUTs").

Highlight LUT 1 by clicking inside the square of LUT 1. The border around LUT 1 will turn orange, indicating that LUT 1 is selected for editing.

Choose the parameters for the SW (i.e. FL1 vs. FSC) by changing the _X_ and _Y_ axes selection on the left display. The LUT will inherit the axes of the left display's plot when axes are changed.

In the left toolbar's sort window function area, choose whether the gate will be a left SW, a right SW, or both, by clicking _Left_, _Right_ or _Both_. Next click _Draw Window_ and draw a SW on the left display. A SW can be drawn as a rectangle, ellipse or polygon.

To draw a rectangular or elliptical SW click on _Rectangle_ or _Ellipse_ and left click and drag on the left display.

To draw a SW of any shape (polygon), single left click on the left display, move the mouse to another position and single left click again and continue the process until the desired shape is drawn.

Right click to close the polygon and finish drawing the SW.

Once the SW is drawn on the left display it will also appear in the LUT on the Right Display if _Show Sort Window_ is checked in the right toolbar.

Data that have a left SW drawn around it will become green, and data that have a right SW drawn around it will become red.

Data that are selected for both right and left sorting will become yellow and will not be sorted.

Additional LUTs can now be selected, and different parameters may be selected for SW drawing. For example, LUT 2 may be chosen to display FL2 vs. SSC.

By drawing another SW around these new parameters it is possible to detect a particle with four desirable characteristics (e.g., FSC, FL1, FL2, FL3; it may fluoresce green, have particular forward and perpendicular scatter values, and not fluoresce red).

In this fashion up to 12 parameters can be set for both right and left sorting (depending on the number of photo multiplier tubes included in the particular instrument configuration).

SWs follow "and" logic; as additional SWs are created in multiple LUTs, only data that is selected in _all_ SWs will be sorted.

Data that are selected for left or right sorting in _all_ SWs will appear green (left sort) or red (right sort) in the left display.

An operator may also choose data outside of a SW to be sorted by again selecting the LUT in the Right Display, drawing a SW in the left display, clicking on _Left_ or _Right_ and clicking _Outside_ in the left toolbar. This allows for "not" logic to be used when multiple SWs are drawn in multiple LUTs.

Overall sort statistics are displayed on the left display, while individual statistics for each LUT are displayed next to each LUT.

LUT statistics report the percentage of particles chosen for left sort, right sort, neither or both for that particular LUT.

Overall sort statistics report the combined percentage of particles selected for left, right, neither or both sort when SWs have been drawn in multiple LUTs.

The _Reset_ button (in the sort window function area) can be used to reset SWs drawn in a particular LUT window by selecting the LUT and clicking on _Reset_.

All LUT SWs can be reset by clicking the _Reset All_ button on the right toolbar). Individual LUT SWs can also be made active or inactive by selecting the LUT and clicking the _Active_ button in the sort window function area. SWs may be moved or stretched by clicking on the _Modify_ button.

LUT Filters

Spigot software allows data filtering based on SWs for easy identification of populations and subpopulations across many parameters. When LUT filters are

activated, the left display will only show data that is inside SWs drawn in the LUTs. LUT SWs can be filtered through in any combination, alone, or not at all.

To apply a LUT filter click the small rectangle of six boxes in the top right corner of the left display. These six squares represent the 6 LUTs and by clicking on one of the squares the data will be filtered through any SW drawn in that particular LUT.

Multiple LUTs can be filtered through in combination by clicking on more of the squares.

Analyzing

Once the system is tuned, data may be analyzed. Spigot provides real-time analytical tools described in the Spigot Interface chapter.

For detailed statistical analysis Spigot allows data to be stored in the *.fcs file format that can be read and manipulated with a separate analysis program.

Save *.fcs files

To save data to an *.fcs file from Spigot, refer to **LIST TOOL** and **QUICK LIST** sections in the Spigot Interface chapter.

Compensation

The instrument is capable of unlimited real-time hardware compensation for up to 16 parameters.

Once compensation values have been applied the user may display, gate on, and store both compensated and uncompensated data.

Compensation is intended to be used strictly with log amplified data.

There are many different methodologies used for compensation. This section will describe two that are suited to the instrument:

- Offline compensation
- Real-time compensation

Both methods begin with the same step: setting PMT gains.

Set PMT gains for the experiment

Run negative (unstained) control particles. Decide which (fluorescent) parameters are going to be compensated (based on your experiment / sort), these will be the parameters for which the PMT gain will need to be accurately set.

Alternatively, you may run single color samples mixed with negatives. This method can be advantageous when setting PMT gains for fluorescence channels excited by lower power lasers since negative populations can be harder to identify.

Plot the first fluorescent parameter and adjust the PMT gain so that the mean of the negative population is visible on scale, but most all of the data falls below the first decade.

For lower power lasers, such as the 635 nm laser, it may be difficult to find a mean for the negative population. In this case set the gain so that most of the data falls in the first decade.

Use the oscilloscope to monitor the data pulses when setting gains to ensure that gains are not set so high that PMTs are saturated (baseline will come up when PMT is saturated).

Continue to plot fluorescent parameters that will be used and set the PMT gains for each parameter as described above.

Note: If using *Method 2 (offline)* and running positive single colors with negative cells, save an *.fcs file for each positive sample that will be used to compensate.

Once PMT gains have been set it is very important that they *not be altered while or after compensating*, as compensation values are directly dependant on PMT gain.

Save an *.sco file so that PMT gains can be recalled if inadvertently altered.

Method 1: Use Spigot to Compensate in real-time

Once PMT gains are set Spigot may be used to compensate data in real time. Use the following procedure for each fluorescent parameter to be compensated.

First, choose parameters that are to be compensated by checking in the comp boxes in Spigot's *Global Options* menu for each channel that will be compensated.

Run a tube of single color control cells.

In Spigot's Left Display plot the control cell fluorescence parameter to be compensated vs. another fluorescence parameter.

In the box next to the parameter drop down dialog click *Compensate* for both parameters so that compensated data will be plotted.

Change the compensation values next to the check box until the desired compensation is achieved.

Continue to plot the single color control against all the remaining parameters, adjusting the comp values for each plot.

Repeat this process, running each of the single color controls and plotting and compensating them against all other parameters.

The full compensation matrix can be viewed in Spigot's COMP menu. Continue until all positive samples have been run and compensated out of all negative parameters.

Method 2: Use third party software to Compensate offline

The compensation matrix may be determined offline by saving single color *.fcs files and third party software to analyze the data and compute the matrix.

Follow instructions provided by the analysis software help files to create a *.mtx (XML) compensation matrix file.

After creating and saving a *.mtx file in the file may be loaded into Spigot by clicking on **LOAD XML FILE** in Spigot's **COMP** pane.

Browse to the saved matrix file and load the compensation matrix. Matrix values may be altered after loading as described in method 1.

Droplet Formation and Sort Streams

Droplet formation is achieved by using a piezoelectric element to focus an acoustic wave into the stream at the nozzle tip.

The stream may need to run for about 10 minutes before droplet formation will become stable.

Most settings for drop formation will be made form Spigot's **SORT CONFIG** pane, and this section will assume that the **SORT CONFIG** pane is open.
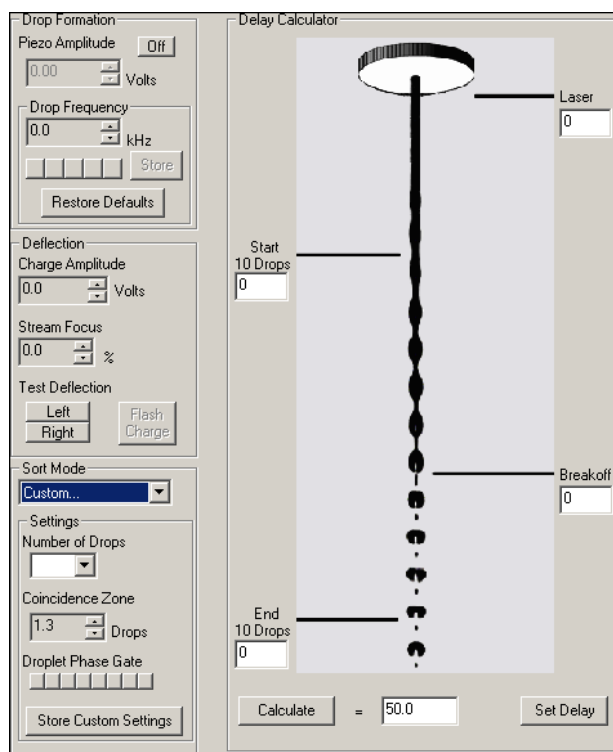
Figure 3.14: Spigot's Sort Config Pane

Open a Spigot Configuration File (*.sco)

Droplet formation settings can be stored in *.sco files. If you have already saved proper droplet formation settings, simply recall them from a *.sco file.

Note that after opening a *.sco file you must choose _YES_ when prompted to load droplet formation settings.

If you do not load a *.sco file, see the steps below for setting up droplet formation.

Choose a Droplet Frequency

Set the *Drop Frequency* according to the pressure and tip size being used (see Appendix B for approximate optimal settings).

Turn on the Piezo drive

If not already on, click the Piezo Amplitude *ON*.

View the Break-Off Point (BOP)

Adjust the micrometer of the drop camera so that the BOP can be seen in the drop monitor.
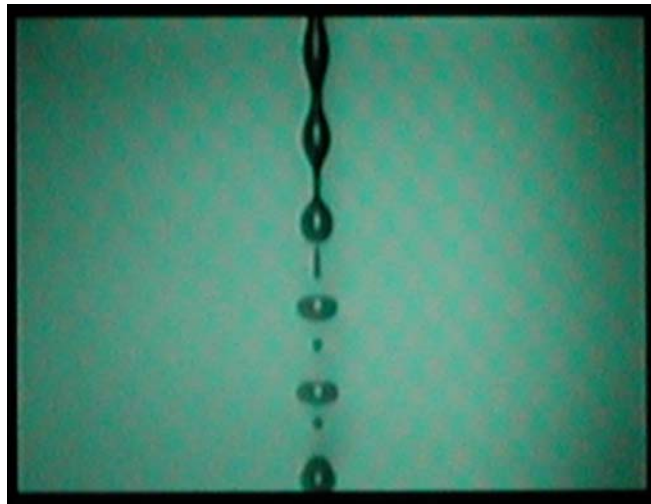
Figure 3.15: Drop camera image

Adjust the Piezo Amplitude

Adjust the *Piezo Amplitude* until the desired BOP is achieved.

Setting the *Piezo Amplitude* too high may cause forward scatter signal CVs to increase, while setting the amplitude too low may cause sort streams to spray.

Experiment with different piezo amplitudes / BOPs to find the optimal setting.

Optimize Droplet Rate

	Note
⚠️	**The piezo rate that forms stream perturbations with a wavelength equal to approximately 4.5 stream diameters is optimal for breakoff stability.** *Petersen et al, Cytometry A 56A:63-70, 2003*.

Adjust the *Drop Frequency* to scan frequencies near the suggested optimum frequency for the pressure and tip size being used.

Choose a frequency that causes the break off point to be as high (short) as possible while maintaining the desired drop spacing. It may be necessary to adjust the camera height and piezo amplitude during this procedure so as to keep the BOP visible in the drop monitor.

Up to 5 pre-set *Drop Frequency* settings can be saved for easy recall with the buttons underneath the *Drop Frequency* box.

Clean, Close and turn on Plates

	WARNING
⚠️	**Make sure that the plates are off by clicking on PLATES on the Monitor Stand. Failure to do so could result in shock when cleaning the plates.**

Clean the deflection plates by wiping them with a kimwipe or other towel wetted with DI water.

Wipe the plates again with a dry wipe to ensure that there is no fluid on the plates before closing them.

Swing the deflection plates into their closed position. The attached magnetic latches will hold them in position.
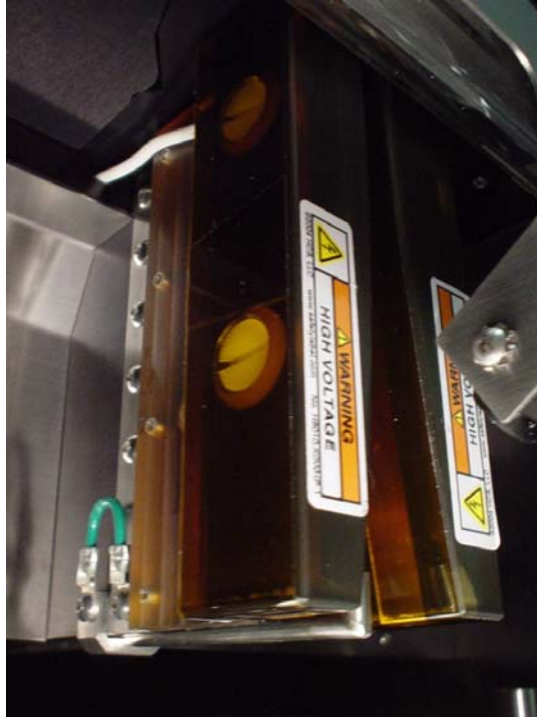
Figure 3.16: Deflection plate asembly

Close the sort cavity door and switch on the high voltage plates by pressing the **PLATES** button on the Monitor Stand.

Note that the button will illuminate to indicate that the plates are on.

Optimize Break Off Point

Follow these steps for creating test sort streams and tuning the piezo amplitude to optimize the BOP. Optimizing the BOP ensures that the drop charge is in phase with the drop formation, which leads to the highest recovery of cells when sorting.

Ensure that the waste flask is centered by clicking the *Safe* button in the **SORT TRAY** pane.

Return to the **SORT CONFIG** pane.

Create left and right test sort streams by clicking on *TEST LEFT* and *TEST RIGHT*. Sort streams should now be visible in the sort stream monitor if the *Charge Amplitude* is set properly.

Adjust the *Charge Amplitude* so that the sort streams clear the drain as shown (Figure 3.17 below).

Note: Too much charge amplitude will cause the sort streams to hit the plates, while too little will not allow the sort streams to clear the drain.
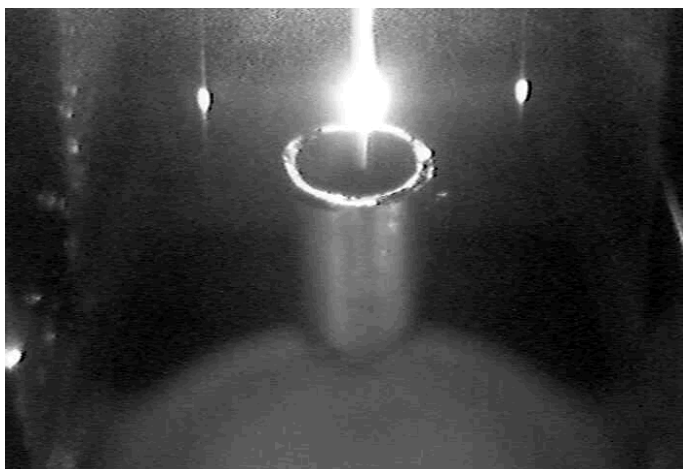
Figure 3.17: Sort stream image, with sort streams

Phase the droplet formation with the drop charge

Use the following method to align the break off point with the drop charge. This will provide optimal immunity against small fluctuations in the breakoff point.

With the _Test Left_ and _Test Right_ buttons on, click on _FLASH CHARGE_.

Adjust the _Piezo Amplitude_ until the sort streams are maximally deflected, with the _FLASH CHARGE_ on. Ignore small satellite streams near the main waste stream when the **1-1 ½** switch is up.

Use the drop monitor to note that the piezo amplitude can be increased or decreased until the next or previous drop is deflected.

Once the desired drop is deflected optimally carefully note the appearance of the droplet formation at the BOP.

View and note the connectedness of the "ligament" or small detached droplet after the last connected drop.

It is crucial that the droplet shape remain constant while sorting. It is sometimes necessary to adjust the _Piezo Amplitude_ while sorting to keep the shape of the jet at the BOP (connectedness of ligament / presence of small satellite drop) constant.

Note that F11 and F12 keys are shortcuts for increasing and decreasing the piezo amplitude.

Turn off _FLASH CHARGE_.

Set CHARGE DEFLECTION amplitude

The proper setting of deflection amplitude results in a sort stream that falls in the center of a sort tube during 2-way sorting.

Place a tube in the left receptacle of the WDU insert. Place a microscope slide over the tube. From Spigot's **SORT TRAY** pane send the WDU to the **2-TUBE**⇨**SORT READY** position.

Quickly click the _TEST LEFT_ button on and back off again to put a small droplet of water on the slide.

Send the WDU to _Present_. Check the slide to see if the droplet would have landed in the center of a collection tube. If not, adjust the _Charge Amplitude_, and/or use Spigot to offset the sort ready position and repeat this test until the stream is deflected to land in the bottom of a sample collection tube.

Alternatively, or in conjunction to altering the _STREAM DEFLECTION_ amplitude, the sort ready position for each sort procedure may also be offset and saved in Spigot on the **SORT TRAY** screen.

If 96-well sorting is desired, follow a similar procedure except use a 96-well tray insert and the _96-Well Sort_ mode for the WDU mode.

Note that you may wish to minimize the stream deflection when sorting into multi-well plates in order to minimize the deflection angle. This will help to ensure that sort droplets will land in the well and not on the sides of wells.

Set STREAM FOCUS

After turning off **FLASH CHARGE**, but with the left and right test streams still enabled, view the sort stream monitor and adjust the _Stream Focus_ until the main center stream (stream going into drain) is as tight as possible (minimize satellite streams).

Drop Delay

Setting the proper drop delay is critical for sort purity and recovery.

When the same nozzle tip and sheath pressure are used each day the delay setting will be very similar day to day, and thus some of the following steps may not be necessary.

The following instructions describe how to find the proper delay setting without knowing the previous days setting.

	NOTICE
⚠️	**It is very important that the fluid stream has had at least 30 minutes of warm up time to stabilize. It is also very important that there are no bubbles in the nozzle. Refer to the Stream Generation section to make sure that the nozzle is properly purged of bubbles.**

Optimize droplet formation

If necessary, re-optimize the BOP as described in the above section on Droplet Formation.

Adjust the _Piezo Amplitude_ as necessary to ensure that the waveform of the jet at the BOP remains constant throughout the delay calibration process.

Minor adjustments to the piezo amplitude are normal, if a major adjustment is

necessary then check to make sure that there are no bubbles or clogs in the nozzle.

Use Spigot to approximate Drop Delay

Open the **SORT CONFIG** screen in Spigot. Scan the drop camera to the points indicated, using the bottom or top edge of the drop monitor as a reference point.

Enter the value of the **DROP POSITION** (indicated on the Pressure Console) for each position.

Figure 3.18: Drop Position readout on Pressure Console

Click *Calculate* and Spigot will calculate the approximate drop delay.

Click *Set Delay* in Spigot to set the system drop delay to the calculated value.

Note that the delay will not be set until *Set Delay* has been clicked.

Run test series with the WDU

Run calibration particles at a low rate (100-1000/sec) and draw a SW around them as described in the Sort Gating section.

From the **SORT TRAY** screen in Spigot send the WDU to *Present*.

Place a microscope slide in the WDU insert in the first position. Send the WDU to *Calibration*⇨*Sort Ready*.

Choose the number of particles to be sorted for the calibration run (defaults to 20). It is recommended (and these instructions will assume) that the sort count be set to 20.

Open the **SORT TRAY** screen in Spigot and click *Sort!*. The WDU will place a 5X5 matrix of puddles on the slide. Each puddle on the slide represents a sort command for the set number of particles (20) at different delay settings.

After the WDU deposits the 25 puddles a screen will pop up that lists the delay setting Spigot used for each of the 25 puddles.

Take the slide to a fluorescent microscope and count the number of particles in each

puddle. A Drop Delay Worksheet is provided in the appendix to this guide as a suggested method for keeping track of the particle counts per puddle.

Identify the three puddles in series with the highest particle count. Take note of the position of the middle puddle of the series of three with the highest count.

Read the delay value from the Spigot delay setting popup that corresponds to the puddle indicated above. This will be the delay setting used for the next calibration run. Set Drop Delay

Click on the proper delay value in the pop-up dialog and click _OK_ to set the drop delay, alternatively the delay may be set from the **SORT CONFIG** menu by entering a delay value and clicking _Set Delay_.

Repeat Delay Calibration

Repeat the above steps until two calibration series in a row yield the same value for the delay. This will be the delay setting used for the rest of the day.

Remember that the sheath pressure, droplet frequency, and BOP all need to remain constant in order for this delay setting to be accurate. If any of those settings change the delay will need to be reset as described above.

Sort Modes

When sorting, it is important to consider both coincident particles and the position of particles inside droplets. Coincidence occurs when two or more particles are closer than the spacing of the droplets, so that more than one particle ends up in the droplet.

Obviously, these droplets must not be sorted in order to achieve maximum purity.

Coincidence is higher when sample rates are higher and when droplet rates are lower; thus, choosing a proper sort mode depends on those settings and the desired result of the sort: purity or recovery.

When the exact count of a sort is important, such as when sorting one particle into each of 96 wells, it is important to consider the position of the particle inside the droplet since particles near the droplet boundaries may end up in either droplet, and empty droplets may inadvertently be sorted.

The instrument sorter provides circuitry to take care of these issues. Coincidence circuitry allows the user to set a coincidence window (in number of drops) before or after an event is detected.

If another particle is detected within the coincidence window, the particles will not be sorted. A 1 ½ drop sort mode is also available.

The 1 ½ drop sort mode will sort two drops when a particle is on a droplet boundary and one particle when a droplet is in the center of a drop, ensuring an accurate sort count.

Phase Gate criteria may also be set. Phase gating allows the user to set the instrument to sort only particles that are in a certain position in the droplet, such as the middle.

This mode may be useful when sorting large particles.

BD recommends four different combinations of these settings to arrive at useful sort modes depending on the desired sort outcome of the sort.

These modes can be chosen from Spigot's **SORT CONFIG** menu.

Other settings may be experimented with as desired by choosing _Custom_ and entering desired values for the coincidence window, number of drops sorted and phase gate criteria.

Important Sort Mode Definitions

Term	Definition
Recovery	The number of particles sorted out of the number requested to be sorted.
Yield	The number of desired particles sorted out of the total number of desired particles in the entire sample aliquot.

Setting 1: Purity-Yield

High Throughput, High Purity, High Yield, Lower Recovery

Use this setting when sample rates are high compared to droplet rates, and when the count (Recovery) of the sorted particles is less important.

> Pre and Post Coincidence = 1 drop, 1 drop sort

> Phase Gate = All

Setting 2: Purity-Recovery

Low Throughput, High Purity, Lower Yield, High Recovery

Use this setting when sample rates are low compared to droplet rates, and when the count of the sorted particles is important.

This mode is most often used for single cell sorting into multi-well plates, and can also be useful when sorting large particles.

> Pre and Post Coincidence = 1 ½ drop, 1 ½ drop sort

> Phase Gate = All

Setting 3: Enrich

Low Throughput, Lower Purity, High Yield, High Recovery

Use this setting when high yield is desired and purity is not as important.

The purity of a sort with coincidence disabled is completely determined by the sample to droplet rate ratio, and may be quite compromised at high sample rates.

This mode is most often used for collecting rare populations where sort yield is more important than purity.

Pre and Post Coincidence = Disable Coincidence, 1 ½ drop sort

Phase Gate = All

Setting 4: Large Particle Recovery

High Purity, Lower Yield, Higher Recovery for Large Particles

This Setting will apply a phase gate that allows only particles in the center of droplets to be sorted.

This is particularly useful when recovery of large particles (greater than ¼ of the nozzle diameter) is important as large particles near droplet boundaries cause problems in the BOP and may cause sort streams to spray.

Pre and Post Coincidence = 1, 1 drop sort

Phase Gate = Middle 4 of 8

Sample Collection

The WDU feature allows sample to be collected into 5 ml sample tubes and 96-well plates. Additional collection trays can be developed for special configurations.

Make sure that the system is aligned, droplet formation is stable, and that the delay setting has been calibrated before collecting sample.

Running Samples

When running samples it is critical that the sample line be back-flushed for about 10 seconds when switching to new samples to minimize sample carryover.

If sample carryover is a major concern, load a sample tube with sheath fluid and run clean sheath through the sample line at a high sample pressure (~3 PSI over the sheath pressure – hold boost button) until no particles are detected.

Alternatively, a new sample line can be installed with ease, ensuring no sample carryover.

Left and/or Right Sort

To collect up to two sample populations simultaneously, use the *2-Tube* WDU sort setting.

To ensure that the sort streams will fall into the collection tubes you may wish to double check the setting of the *Piezo Amplitude* and the *Charge Deflection* amplitude as described in the droplet formation section.

Load two 5 ml sample tubes into the WDU insert. Send the tray to the *2-Tube* ⇨ *Sort Ready* position.

Make sure that the **PLATES** are on . Choose the number of cells to be sorted left or right from the **SORT** screen.

Click on *Left Sort* or *Right Sort* to begin sorting (based on your gating scheme) into the collection tubes. A progress bar will pop up in Spigot that will give feedback on the

number of cells sorted as the sort progresses, as well as an abort rate indicated near a circular red icon.

Use the monitors during sorting to ensure that particles are being sorted and that the BOP remains constant.

A sort may be paused or terminated from Spigot.

When the sort is done the WDU will place the tray in the _Safe_ position. The tray can then be presented and sample tubes can be collected.

96-Well Sort

96-well sorting allows a particular number of particles to be placed into each of the wells of a 96 well plate.

Follow directions above but use the _96-Well_ mode for all tray settings.

Remember that the sort ready position may be offset to ensure that the sort streams will fall into the center of the wells.

Shutting Down

Close Spigot

Shut down Fluidics

For optimal results and decreased chances of contamination, the instrument fluidics should be shut down, emptied out and allowed to dry overnight.

Use the following shutdown procedure:

Allow sample line to back flush for at least 1 minute. Meanwhile, clean the outer sample line with a syringe filled with filtered or DI water. Place the flush bucket in the sort chamber.

Close all fluid valves by clicking **RUN** off.

Turn off sheath pressure by flipping the **AIR** switch down on the Pressure Console.

Optional Step: Connect a 50ml syringe filled with 2% bleach or a dilute surfactant to the Luer connector on the sheath line. Switch on the **AIR**, press **RINSE**, and flush the sheath and waste lines. Deactivate the **RINSE** mode, reconnect the sheath lines, and switch off the **AIR**. Continue as below.

Empty sheath reservoir, rinse and fill with about 200ml of 0.22mm filtered distilled or de-ionized water.

Reattach sheath reservoir and flip on the **AIR** switch on pressure console.

Click **RINSE** and **BACKFLUSH** to let the clean water flow through the system until it has emptied the sheath reservoir and the system blows air. Allow the system to blow air until no more water is coming up the sheath line and until no more water drips from the sample line.

Click **RUN** off, remove nozzle tip (put the dry nozzle tip in a safe place), place flush bucket under nozzle assembly. Click **RINSE** on and let system blow for 10-15 minutes to fully dry out tubing.

Flip the **AIR** switch off on the pressure console.

Remove sheath and waste reservoir and empty all liquids. Rinse and or clean reservoirs as necessary.

Turn reservoirs upside down on the table on a piece of absorbing tissue. Be careful not to damage gauges or fittings.

Clean Sort Chamber

Open and clean the plates using a wet kimwipe.

Clean away any salt build up on the drain, in the sort chamber, and at the sample station.

Shut down Electronics Console

Turn the Electronics Console off by flipping the **AUXILIARY POWER** switch on the bottom left of the console to the **OFF** position.

Turn off all switches on the Control Panel.

Shut down Lasers

Shut down all lasers as specified by the manufacturer. Typically this involves shutting off key switches and powering down each laser.

4

Spigot Interface

Summary

This chapter describes the layout of the Spigot interface and the various options available to the user within each of the various screens.

Overview

The Spigot interface consists of three main components: the toolbars and the left and right displays (see below). The size and position of these three components on the screen cannot be changed.

The left display is fixed in place, and it always contains a two-dimensional (2D) data display with corresponding histograms. Particle rate, sort percentages and other data are displayed here as well.

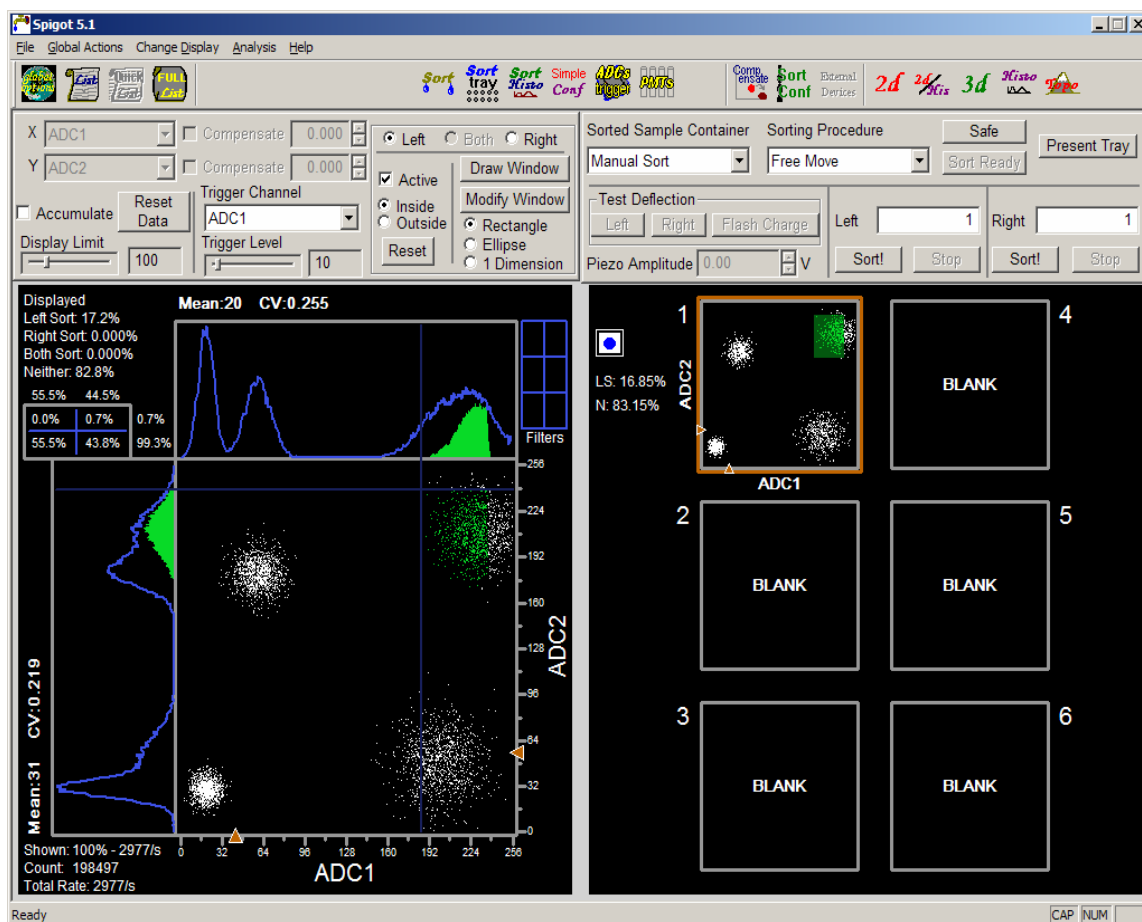The right display can show a variety of configuration and data analysis screens.

Figure 4.1: Spigot Display

Left Display💬

The left display consists of two main components: 1) The 2D real time dot plot (black area of the screen and 2) the control dialog (gray area of the screen).
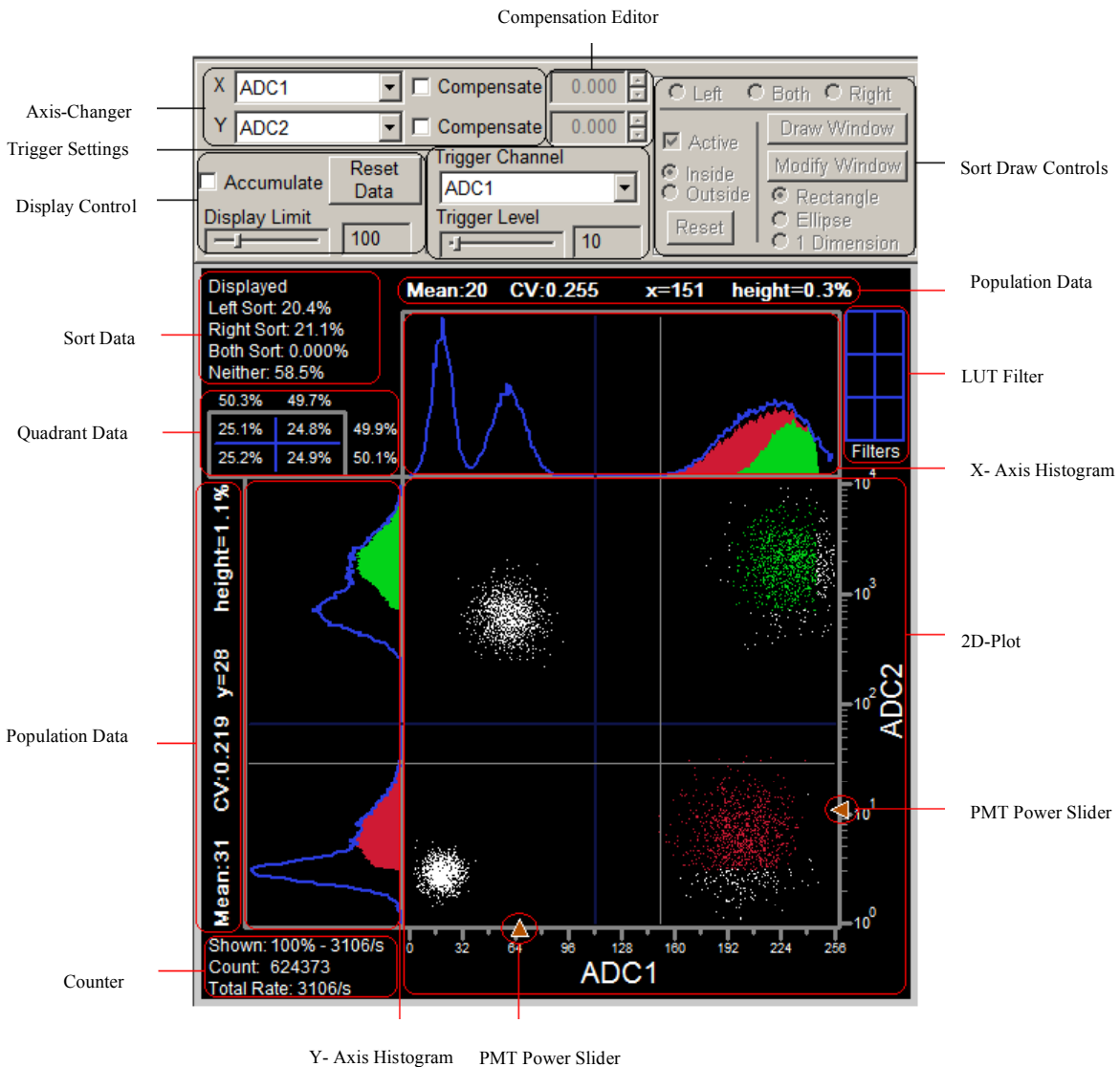
Compensation Editor

Figure 4.2: Left Display

2D real time dot plot:

Each dot represents data (a cell) the sorter has received from its photo multiplier tubes (PMTs) as processed by the analog-to-digital converters (ADCs). This data represents the intensity of light re-emitted by particles in the sample stream upon laser excitation, after the emitted light has passed through the instrument's filter set.

The default color of the dots displayed in the 2D plot is white, and they change color depending on whether they fall in gates corresponding to the left (green), right (red), or both (yellow) sort directions.

The axes of the plot can be changed from linear scale to log scale (see 0 for details). The axis scale labels can be switched off by clicking on the axes.

Cross-hairs can be used to create quadrant statistics and measure population statistics.

LUT filters

Data in the 2D-Plot can be "filtered" using the LUT filters. The LUT filter contains six squares that represent the LUTs used to draw sort gates. By clicking in the LUT filter square corresponding to a LUT with a sort gate,or "Sort Window", the user can filter the data so that only data that falls into that sort gate is viewed in the 2D-Plot.

Multiple LUT filter squares can be checked in and the data will be filtered through all of those corresponding sort gates.

This tool is especially helpful when trying to identify sub-populations and can be used to eliminate cells that the user is not interested in from the 2D-Plot.

For example, if the user has drawn a sort gate on scatter parameters that includes lymphocytes in the first LUT, then by clicking in the first LUT filter only lymphocytes will be shown in the 2D-Plot, regardless of the axes plotted.

Also, Sort Data will reflect LUT filtering and can be useful when percentages of gated populations are needed.

Note that sort decisions are not affected by LUT filtering.

Sort Data

Percentages of particles displayed as falling into the left and right SWs are displayed in the upper left hand corner of the left display.

Clicking on that portion of the screen toggles the displayed data between percentages and particles/sec. This data will change with application of LUT filters.

Histograms

The histogram is also correspondingly colored so that the user can quickly recognize how the distribution of points falls within each sort gate.

Mean Channel and Coefficient of Variation (CV) information for each histogram are also displayed.

When moving the mouse pointer over the dot plot, the relative height of the histogram at that point will be displayed.

Quadrant data

If the user clicks the left mouse button while positioning these cross-hairs, blue lines will be placed in the plot dividing it into quadrants.

The numbers of particles falling within each quadrant is displayed in small blue boxes in the upper left area of the display.

Counter

A particle counter is available by left-clicking the rate region or using the keyboard shortcut **CTRL-SPACE** and functions similar to a stopwatch. Particle injection rates are shown in the lower left hand corner.

PMT Power Slider

The PMT power slider is used to increase the power of the PMT attached to the channel. It is only visible if the PMT attached to the channel is on.

Population Data

The cross hair data includes mean, covariance, height in percentage of the highest population peak and position based on the current position of the cursor.

Control Dialog

Axis Changer

The user can select which parameters to display on the X and Y axes of the left display by choosing from available parameters from the drop-down lists labeled X and Y on the left side of the toolbar.

Clicking on the compensation check box will display the compensated channel shown in drop-down lists labeled X and Y.

The compensated version of any channel is labeled "*" followed by the channel name (e.g. the compensated version of channel "X" is "*X").

Compensation Editor

Compensation Matrix can be edited if the channel X and Y are marked for compensation in **GLOBAL OPTIONS** and both the compensation boxes are checked.

The edit boxes right of compensate check boxes display the value of compensation set for the channels displayed in X and Y (the top edit box is the value of X compensated from Y and the bottom edit box is the value of Y compensated from X).

Trigger Settings

The system trigger can be selected and trigger level (threshold) set using the trigger settings that consist of a drop down list for selecting the *Trigger Channel* and a slider control for setting *Trigger Level* for the trigger channel.

Sort Draw Controls

For sorting operations, the left display is used in conjunction with the right display's sort screen for drawing sort windows (SWs).

When one of the six LUTs displayed on the right display is highlighted with an orange border, the left display changes to show the same parameters as the highlighted LUT.

Left, *Right*, *Both* radio buttons should be select before drawing the gates to select the type of the gate. Based on *Left, Right, Both* gate selected the color of the dot on which the gate will be drawn in the plot is displayed as green, red and yellow respectively.

Note: Only draw both SWs, if both left and right SW will also be drawn.

Draw Window is enabled only after the gates type is selected.

Clicking on it enable the different gate shapes - *Rectangle*, *1 Dimension* (rectangular that can be varied only in one dimension), and *Ellipse*.

The user can also draw polygons by repeatedly single left clicking on the 2D-Plot until the desired shape is drawn, and then right clicking to complete the gate.

Modify Window provides the user with the option of modifying the shape of the gates drawn for the sort window selected. This feature is available only if there is a gate in the selected (orange border) LUT.

Active, _inside_, _Outside_ are enabled if there is gate in selected LUT. The currently selected gate can be activated and deactivated using the _Active_ checkbox.

The radio buttons determine whether cells must be _Inside_ or _Outside_ the drawn shape in order to match the gate. Outside gates can used to exclude population from the gating scheme.

Reset button reset the gate if any, in the selected LUT. Clicking this button twice will delete both the left and right gates on the current dotplot.

Display Control

Accumulate if checked displays the all events on screen. Use this option to view rare populations.

Display Limit slider controls the number of events to be displayed at a given time on the 2D-Plot. _Accumulate_, if checked, display limit is maximum (infinite).

Reset Data resets the data in the 2D-dot plot and on the histograms.

Right Display

The right display can be customized to show a number of different instrument configuration and data analysis screens. The available screens are:

- Sort Controls
- Sort Controls with Histograms
- Simple Hardware Configuration ("Simple Config")
- Configure Compensation
- Sort Settings
- 2D/Histogram
- 2D Plot
- 3DPlot
- Histograms
- Topographic Histogram.
- Sort Tray

The right display screens can be selected by clicking on one of the colored buttons on the toolbar or via the **CHANGE DISPLAY** drop down menu on the menu bar.

Some of the screens present overlapping data or configuration choices. Users can set the same parameter on more than one screen and other affected screens are immediately updated to reflect the new setting.

Where appropriate the right main section of the toolbar changes to allow user input for customizing the selected screen and setting various parameters. In other cases the right section of the toolbar is completely replaced by the selected screen.

Each of these right display screens are described in more detail in the remainder of this section.

Sort Controls

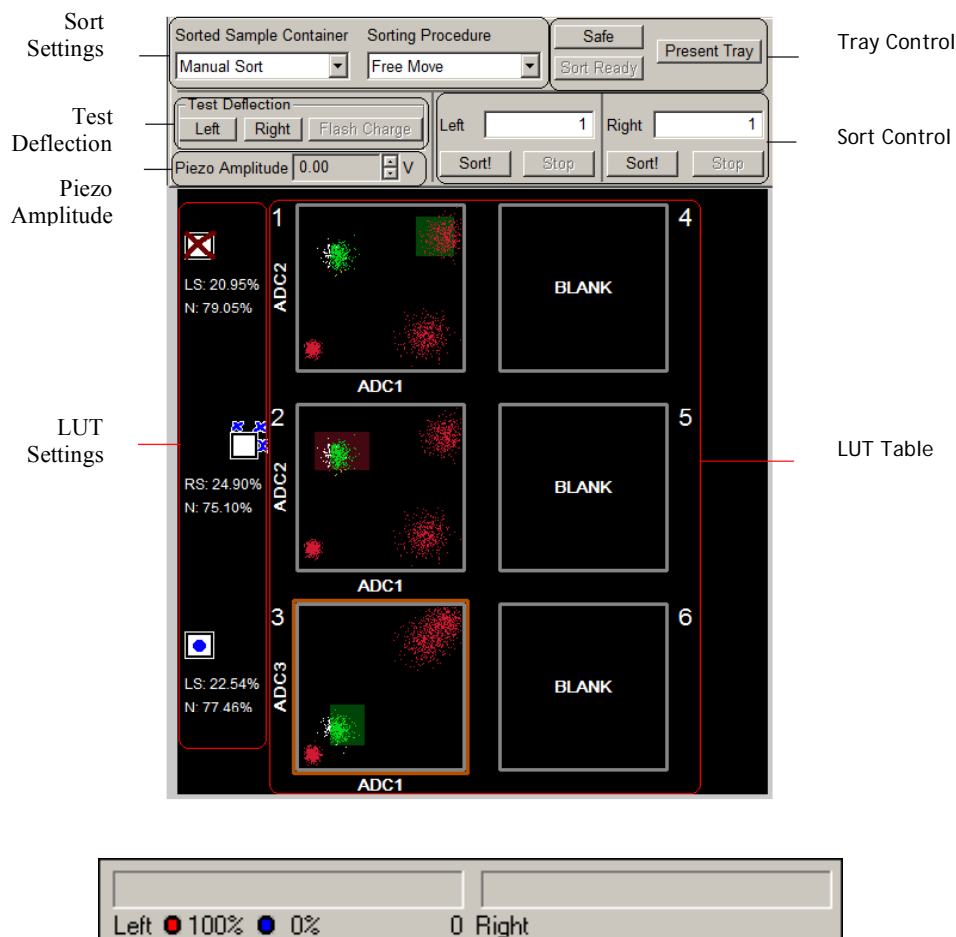The Sort display consists of the components shown below.

Figure 4.3: Sort window (above) and Sort status window (below)

LUT Table

When Spigot is first started, or if **SORT** display is active, the right display shows six numbered squares representing LUTs, and the right side of the toolbar changes to show sorting related options.

Spigot thus enables specifying up to twelve SWs (six left and six right). For each set, the current data is shown relative to the axes in which the SWs are defined.

These smaller displays are updated in real-time similar to the larger dot plot in the left display, and immediately reflect changes made in the SW via the left display.

Dots are also color-coded in these windows in similar fashion to the left window.

Clicking on any LUT immediately brings its contents up on the left display. In this way, users can watch their data in real-time on up to six sets of axes and quickly

switch between these axes on the larger left display.

LUT Settings

Statistics are also displayed for each of the defined SWs as well as current SW status information.

Note that statistics for each LUT are always out of the total cell population, for statistics of sub-populations use the LUT filters.

If a sort gate is drawn in an LUT, a small icon will appear representing that gate. The icon can be clicked on to toggle the active state of this gate, or right-clicked to toggle the gate between inside and outside.

Sort Settings

Sorted Sample Container provides the user with the list of tray types into which the sample can be sorted.

Sorting Procedure defines the way in which the samples are to be placed in the container.

Preset Positions

These are the controls of sort tray.

Safe, *Present Tray*, *Sort Ready* sets the sort tray to a safe position, present the tray for placing the sample holder and places the tray to the first sort position based on the sort settings.

While *Safe* and *Present Tray* are always the same, *Sort Ready* changes based on the *Sorted Sample Container* selected.

Each *Sort Ready* position may be offset to ensure that sorted sample will go into the selected container. Offsets may be saved so that they can be used from day to day.

Test Deflection

Left, *Right*, *Flash Charge* create test deflection streams used for optimization of the break-off point.

Piezo Amplitude

User can Increase and decrease the piezo amplitude. Piezo Amplitide can also be increased or decreased by using *F11* and *F12*, respectively.

Sort Controls

Once the SWs have been created to the user's satisfaction, sort controls are conveniently located at the top of the screen.

The user can instigate left sort, right sort, pause, resume and also terminate sorts.

Sort Status:

Shows the current sort status for both the left sort and the right sort. This dialog appears only when the _Sort_ button is clicked.

The progress bar shows the progress of the sort, while counters give detailed real-time feedback.

The number next to the blue dot represents the percentage to completion of the sort and one next to the red dot displays the abort rate during a sort.

Simple Hardware Configuration

Figure 4.4: Hardware configuration pane

PMT Main Power

PMT Main Power when clicked switches on the PMT Main Power. This is useful when all PMTs need to be turned off when making changes to optical filters.

Scope Display

ADC channels are selected for display on the oscilloscope using the Scope Display control.

If Scope channels should follow left display channel is checked then the channels displayed in the scope are the channels selected or displayed in the Axis Changer of the Left Display.

Event Trigger Delay

There are spin controls for setting the *Trigger Delay* for Events 2 through 5. See chapter 4: Operating Procedures.

Hardware Settings

Setting	Description
Event	Enables user to choose the event for each channel.
Channel Name	Allows channel names to be edited.
Power	Enables user to power the PMT attached to the channel. Clicking on *Power* enables the PMT slider in the left display.
Amplifier Typ	Sets the display of data of the channel to be amplified in logarithmic or linear mode.
PMT Power	Can used to set PMT gains. The PMT gain can also be adjusted by using the PMT slider in left display.

Configure Compensation

Spigot can be configured to change the data display to compensate for spectral overlap.

Clicking **COMPENSATE** changes the right display to that shown below in Figure 4.5. The user can view a "10 X 6" matrix at one time.

The vertical and horizontal scroll bar appears only when the dimensions of the matrix exceed 10 columns or 6 rows.

Channels allowed to be compensated can be added or removed from the **GLOBAL OPTIONS** dialog *(see 0 for details)*. This feature may be useful for removing channels that will not be compensated, for example scatter parameters.

The matrix displayed in the Compensate Menu is for display only. Compensation matrix values can be edited from the left display control dialog.

If compensating channel X from channel Y look at the element that has channel X as column header and Y as the row header.
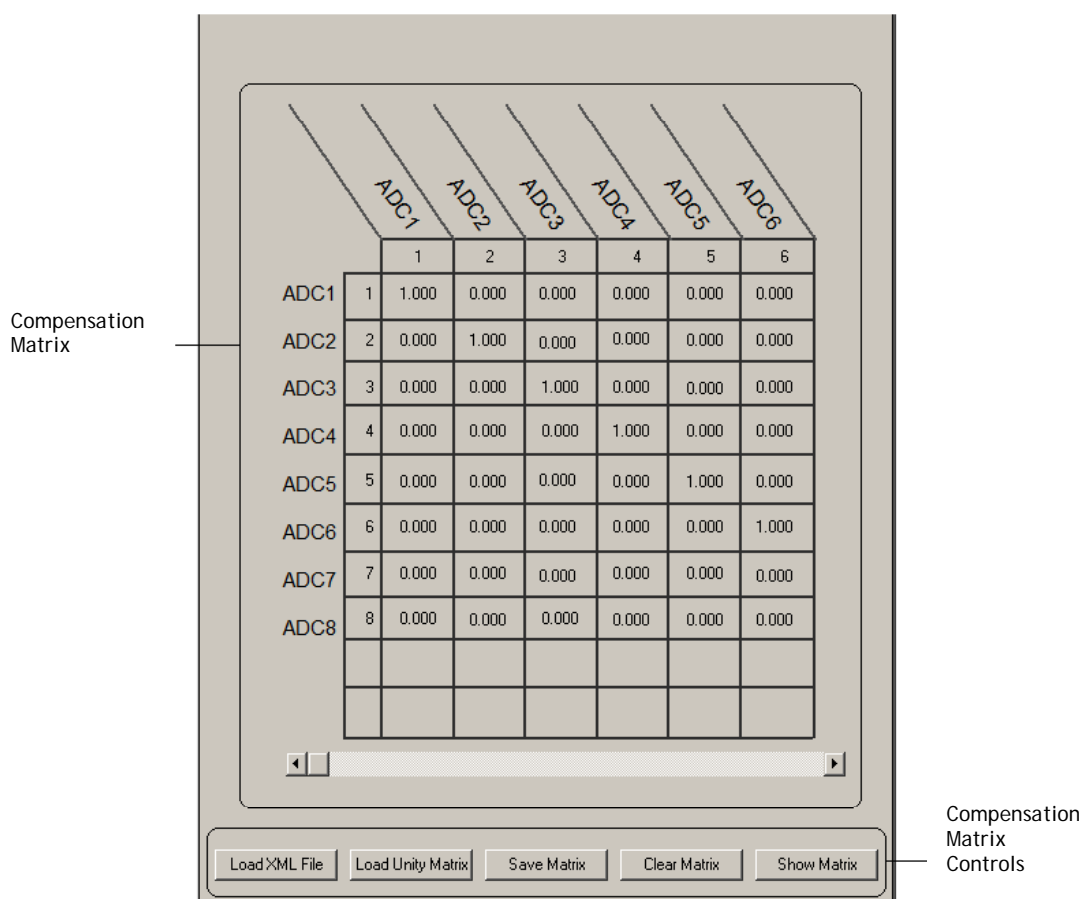

Figure 4.5: Compensation matrix controls

Compensation Matrix Control

| Control	Description
Load XML Fil	Allows the user to load compensation matrix from XML file. It will also pop up a message to assign the values to the respective channels if the channel name in the file does not match the channel name in the current settings. The user will lose all the previous edited values. This feature is useful when third-party analysis packages are used to create compensation matrix files.
Load Unity Matrix	Makes the compensation matrix a unity matrix of size equal to the total number of allowable compensation channels. The user will lose all the previous edited values. This may be useful as a starting point when compensating in real-time in Spigot.
Save Matrix	Provides the user with the option to save the compensation matrix to an XML file. Only the matrix that can be seen in the **COMPENSATE** dialog is save to the XML file.
Clear Matrix	Makes the compensation matrix a unity matrix of size equal to the total number of channels marked for compensation. The user will lose all the previous edited values.

Sort Configuration
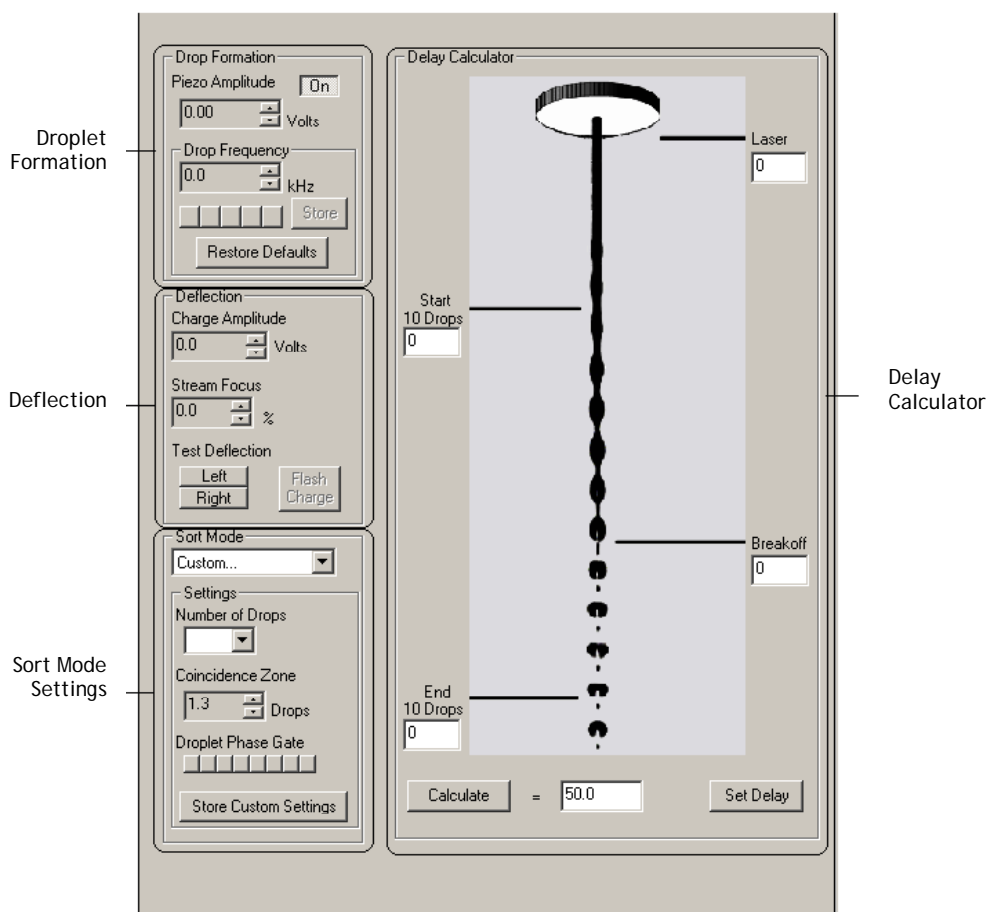
Figure 4.6: Sort Configuration pane

Drop Formation

User can use *Piezo Amplitude* increase and decrease the piezo amplitude. This can also be done by using *F11* and *F12* respectively.

The *Drop Frequency* control is used to tune number of drops per second in your sort.

Deflection

Drop charge amplitude and stream focus can be set using *Charge Amplitude* and *Stream Focus*.

Left, *Right*, *Flash Charge* test deflection streams for optimization of break-off point.

Sort Mode Settings

The user can use this dialog to choose either pre-set sort modes, or to define custom sort modes.

Parameters that can be changed for a custom sort mode are number of drops to sort, coincidence zone and phase gating.

See Sort Modes for more detailed information on sort modes.

Delay Calculator

This dialog is used to estimate the drop delay. There are boxes for entering drop camera values corresponding to four points on the stream, and for entering clock or drop frequency.

Spigot estimates the sort delay setting based on these inputs when the _Calculate_ button is clicked.

The drop delay is set to the hardware when _Set Delay_ is clicked. Values may be entered manually into the delay box, or the calculation method described above may be used.

Note that the drop delay is set in units of number of drops from the laser intersection to the break off point.

See Drop Delay for more information on setting the drop delay correctly.

2D/Histogram View

The 2D/histogram screen permits the display of an additional dot plot window together with histograms on the axes.

This display is independent of the left window. The display and controls are identical to those described in Left Display for the left window.

Figure 4.7: histogram view

2D Plot View *2d*

The 2-dimensional plot screen permits the display of an additional dot plot window. This display is independent of the left window.

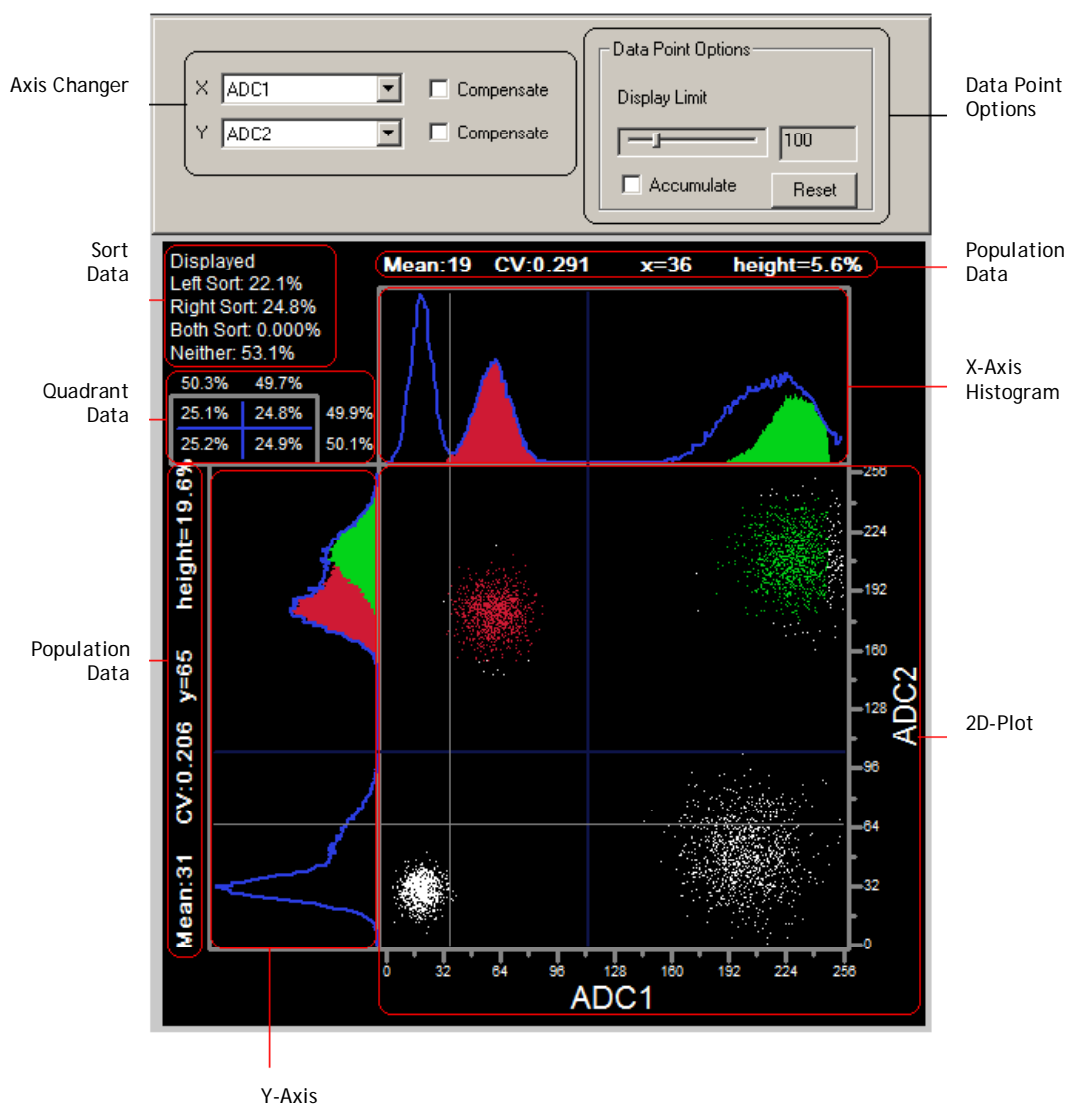The display and controls are identical to those described in Left Display for the left window with the exception of the histograms, which are not shown in this display.
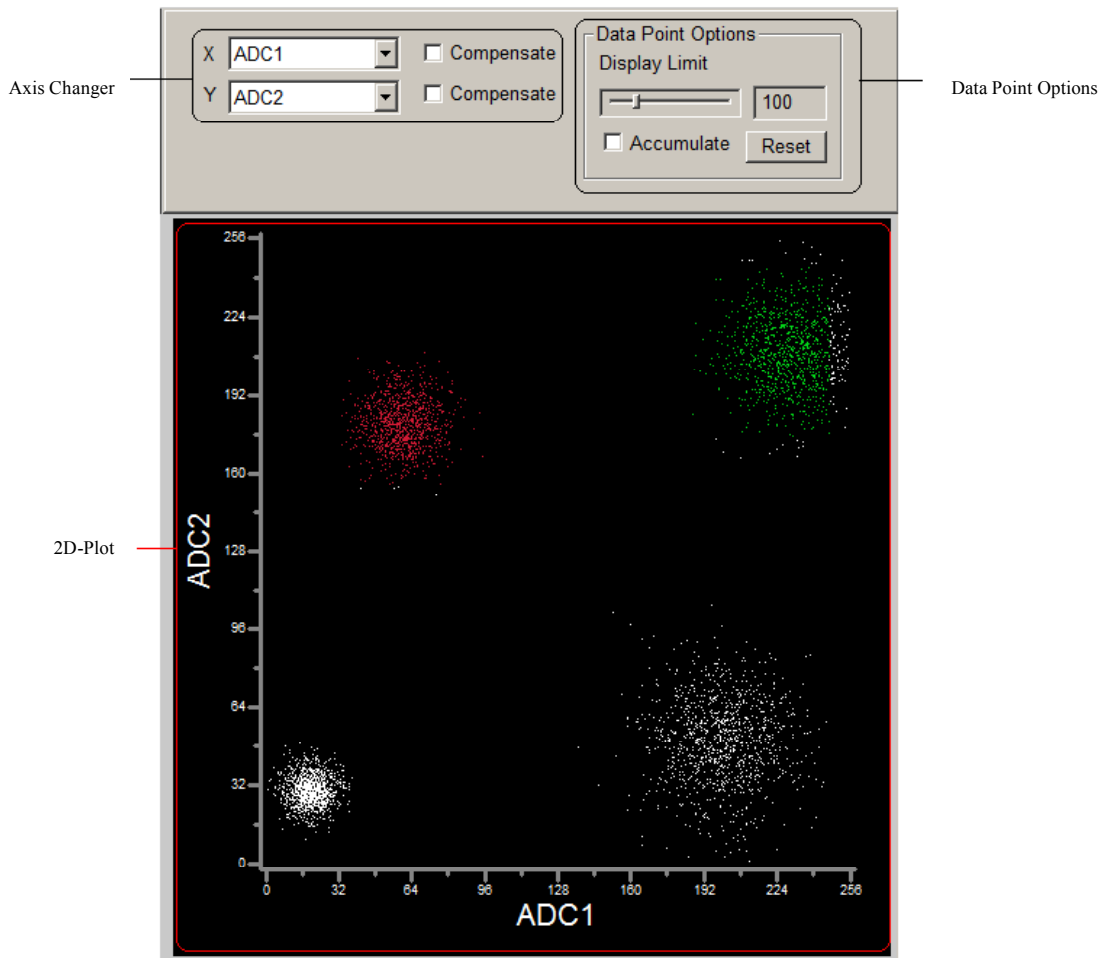

Figure 4.8: 2D plot view

3D Plot View

The 3D Plot View consist of two components:
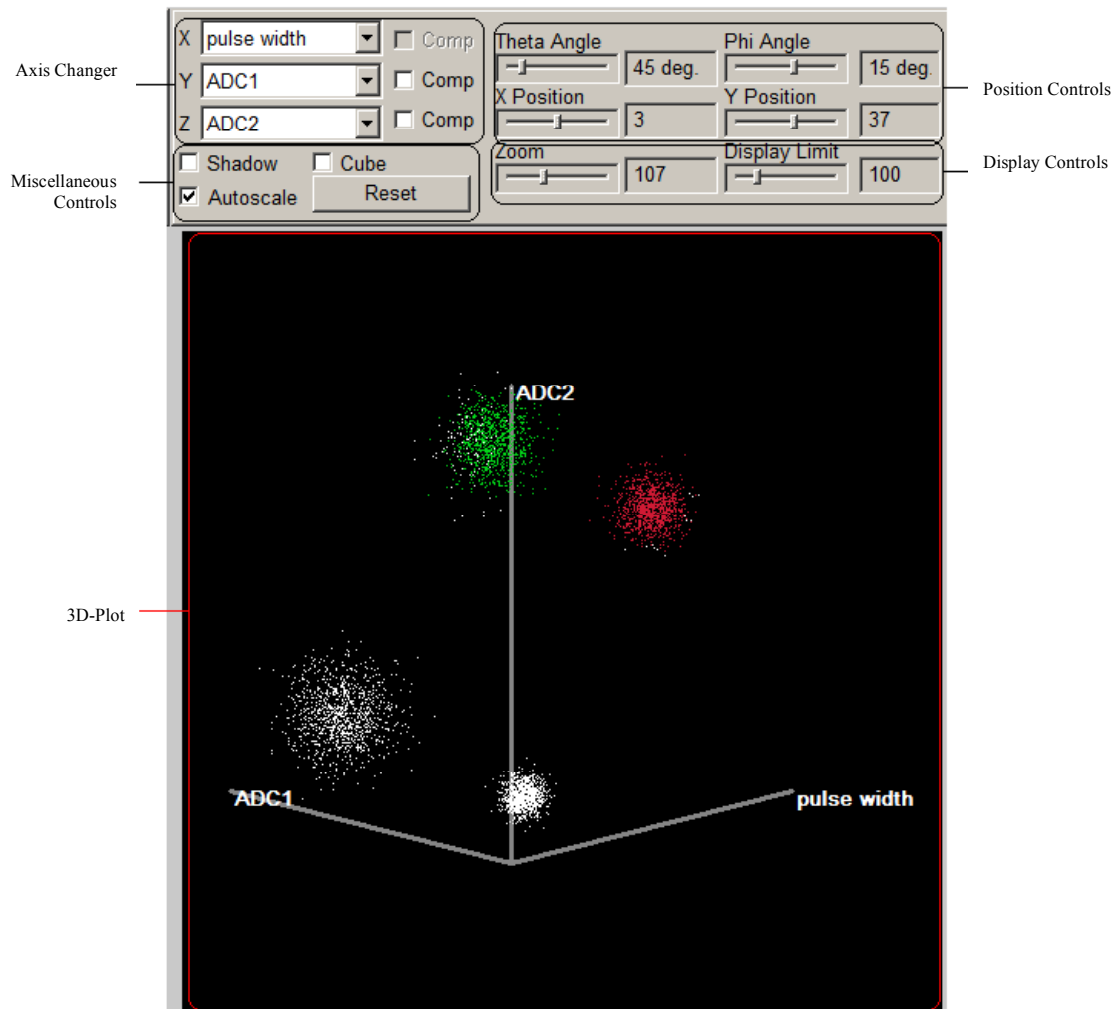
- 3D dot plot
- Control dialog.

Figure 4.9: 3D Plot View

3D-Plot

The *3D* analysis view adds the additional feature of visualizing the data in a three axes perspective dot plot.

Axis Changer

In this view, the right side of the toolbar enables selection of the three parameters displayed.

Miscellaneous Controls

Control	Description
Autoscale	If _Autoscale_ is unchecked, the plot can also be moved in the X and Y directions, and can be zoomed in and out, using slider controls located in the toolbar above the display.
Shadow	If the _Shadow_ box is checked, Spigot draws a colored dot "shadow" on the lower plane of the display.
Cube	If the _Cube_ box is checked, Spigot draws dashed lines to complete a cube, allowing another way to visualize the data in 3D

Display Controls

Controls	Description
Autoscale _Zoom_	If _Autoscale_ is unchecked, the display can be zoomed using the _Zoom_ slider control.
Display Limit	As with most displays in Spigot, the limit of dots displayed can be adjusted using the _Display Limit_ slider control.

Position Controls

Orientation of the display can be adjusted using the _Theta Angle_, _Phi Angle_, _X Position_ and _Y Position_ slider controls on the toolbar (numerical values are shown for reference).

These controls affect the display differently depending on the _Autoscale_ selection.

Histograms View

The 3D Plot View consist of two components:
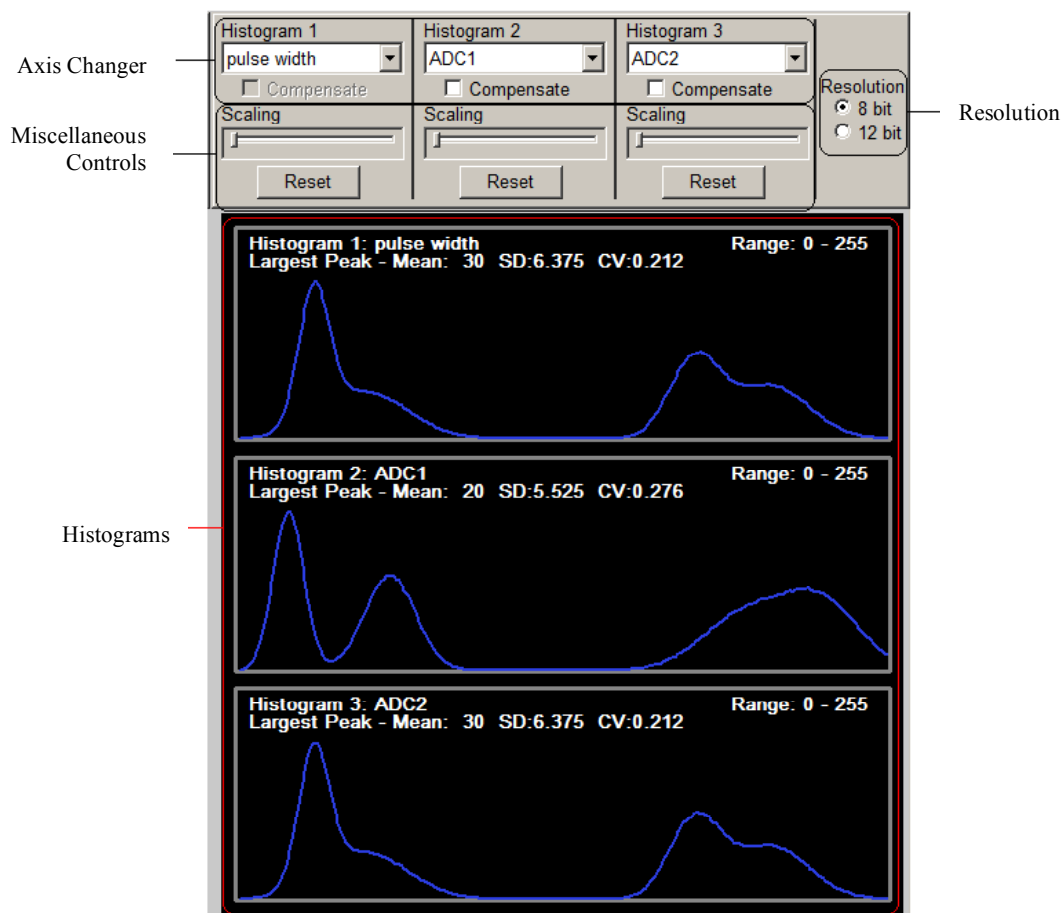
- Histogram plot
- Control dialog.

Figure 4.10: Histogram View

Histogram

In the *Histogram* view, three histograms allow convenient comparison of data from three channels. The histogram name, mean, range, standard deviation and covariance are also displayed.

Axis Changer

The right side of the toolbar has drop-down lists for selecting a channel for each histogram to display.

Miscellaneous Controls

Controls	Description
Reset	Allow resetting of individual histograms.
Scaling	Introduces a digital gain in the histogram.

Resolution

There is also an option to display the data in 8-bit or 12-bit resolution.

Topographic Histogram View

The Topographic Histogram View consist of two components:

- Histogram plot (black area of the screen)
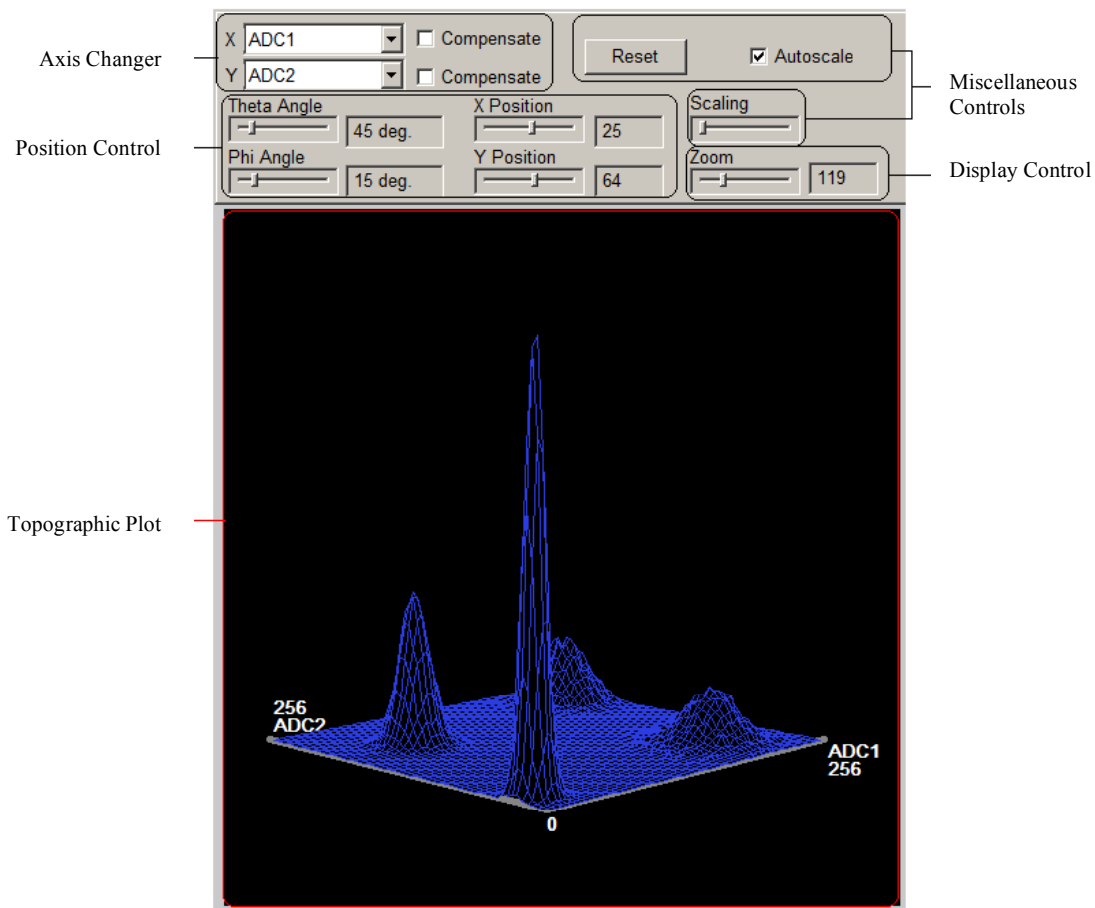- Control dialog (gray area of the screen)

Figure 4.11: Topographic Histogram View

Topographic Plot

This analysis view presents the data in a 3D topographic histogram

Axis Changer

The right side of the toolbar contains drop-down lists for selecting the channels to be displayed on the X and Y-axes of the histogram.

Miscellaneous Controls

Controls	Description
Reset	Allow resetting of individual histograms.
Scaling	Introduces a digital gain in the histogram.
Autoscale	If *Autoscale* is unchecked, the plot can also be moved in the X and Y directions, and can be zoomed in and out, using slider controls located in the toolbar above the display.

Display Controls

If *Autoscale* is unchecked, the display can be zoomed using the *Zoom* slider control.

Position Controls

Orientation of the display can be adjusted using the Theta Angle, Phi Angle, X Position and Y Position slider controls on the toolbar (numerical values are shown for reference).

These controls affect the display differently depending on the Autoscale selection.
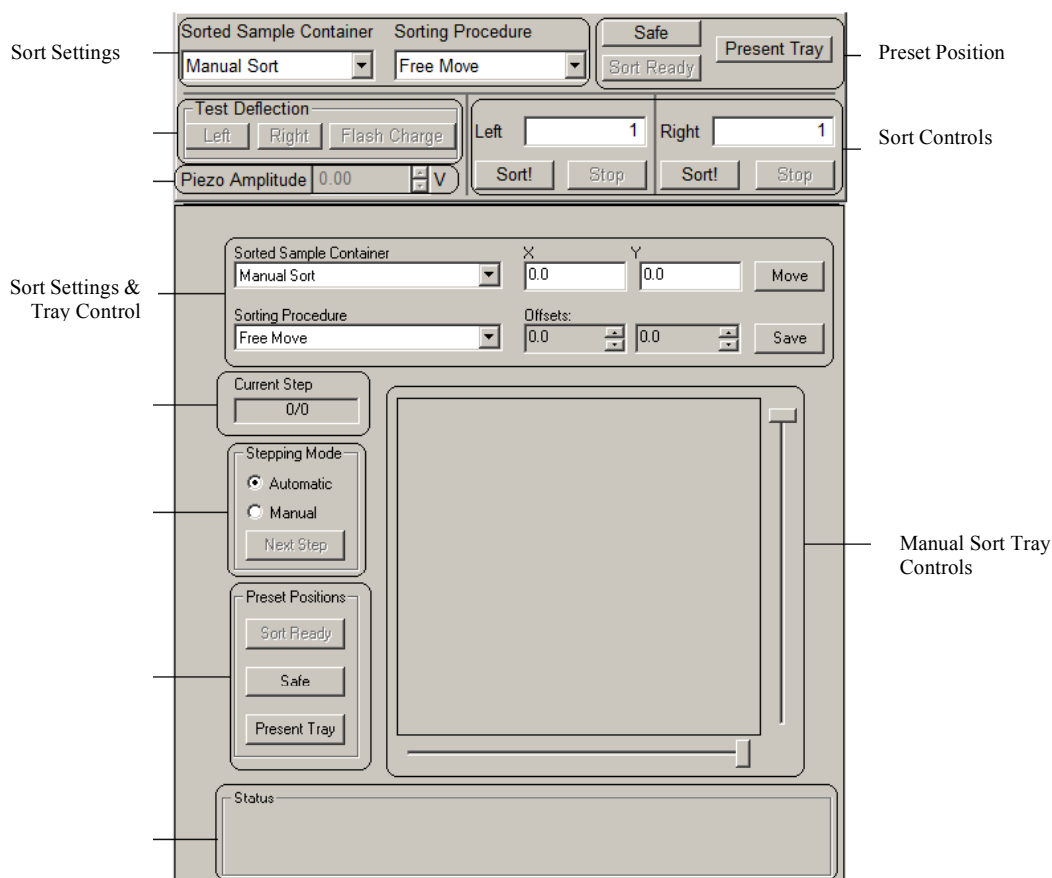
Sort Tray

Figure 4.12: Sort tray configuration

Sort Settings

Setting	Description
Sorted Sample Container	Provides the user with the list of containers into with the sample can be sorted. For example: 2-tubes, 96-well plates, 384 well plates etc.
Sorting Procedure	Defines the way in which the samples are to be placed in the container. For example, during a 96-well plate a different number of cells can be sorted into each well.

Files can be created that define both a list of coordinates (for the sorted sample container) and any number of procedures for that container. In this way any custom sort collection protocol may be created.

Contact BD for details on creating custom files for sort collection.

Preset Position

These are the controls of sort tray. *Safe*, *Present Tray*, *Sort Ready* sets the sort tray to a safe position, present the tray for placing the sample holder and places the tray to the first sort position based on the sort settings.

Each sorted sample container has a unique sort ready position and the tray can be moved to that location by choosing the desired sample container form the drop down list and clicking on sort ready.

In addition, sort ready positions may be offset to ensure that sample will land into the container as desired. Use the offsets to offset the sort ready position up to +-5mm in both the x (left-right) and y (in-out) directions. Note that the origin of the tray coordinate system is in the back right corner, thus to move the tray out is to make a positive change in y and to move left is a positive change in x. Use the Sort settings and tray control described below to offset and save offsets to sort ready positions.

Sort Control

Once the SWs have been created to the user's satisfaction, sort controls are conveniently located at the top of the screen. The user can perform left sort, right sort, pause, resume and terminate sorts. The sort setting and sort tray controls and test deflection will be disabled when the any of one of the sorting operation is being performed.

Test Deflection

Left, *Right*, and *Flash Charge* are used to create test deflection streams for optimization of break-off point.

Test Deflection can be clicked quickly on and off again when a sorted sample container is in place to test the positioning of the container. The sort ready position may need to be offset as described in 0 to ensure that sorted sample will go into the container as desired.

Piezo Amplitude

User can Increase and decrease the piezo amplitude. This can also be done by using *F11* and *F12* respectively.

Sort Settings & Tray Controls

Setting	Description
Sorted Sample Container	Provides the user with the list of tray prototypes into with the sample can be sorted into.
Sorting Procedure	Defines the way in which the samples are to be placed in the container.
Move	Co-ordinates can be typed into the _X_ and _Y_ boxes and the tray will move to the specified location when _Move_ is clicked. Sort ready positions can be offset by +/- 5 mm for both _X_ and _Y_ positions and saved by clicking _SAVE._

Current Step

Current Step displays the current step of the sort tray.

Stepping Mode

The user has the option to move the tray manually step by step when the _Manual_ stepping mode is selected.

Sort Status

Sort Status displays the current status of the sort tray.

Manual Sort Tray Controls

By moving the vertical and horizontal slider in the Manual Sort Tray Control the tray can be moved to any desired position.

Global Options

Spigot may be also customized to fit user preference and instrument configuration by clicking on **GLOBAL OPTIONS** or by selecting **GLOBAL ACTIONS**⇨**GLOBAL OPTIONS** from the Global Actions drop down list on the menu bar. The dialog box that appears is shown below.

Individual ADC channels may be deactivated by clearing the check boxes on the left side of the dialog box. They can be set for compensation by selecting the checkboxes under the _Compensate_ header.

Next, channel names may be changed from the default names ADC1, ADC2 etc. (this feature is also available on the hardware configuration screens.)

The **GLOBAL OPTIONS** tool also allows "remapping" of PMTs to ADCs from the default configuration of ADC1 to PMT1, ADC2 to PMT2, and so on.

Each drop-down list under _Attached PMT_ enables mapping any PMT to a particular ADC, to reflect a change in hardware wiring of ADCs and PMTs.

The PMT types can also be specified in this dialog. PMT-ADC mapping is not necessary if PMTs are attached to corresponding ADCs.

Note: _Mode_ and _Signal Type_ are advanced options and should be set to peak and real respectively, except for special applications. Contact BD for more information on these settings.

Figure 4.13: Global Options

	CAUTION
⚠️	**This feature should only be used by advanced users or technicians as it depends on the instrument's hardware configuration. Spigot and the instrument will not operate properly if this feature is set up incorrectly.**

List Tools
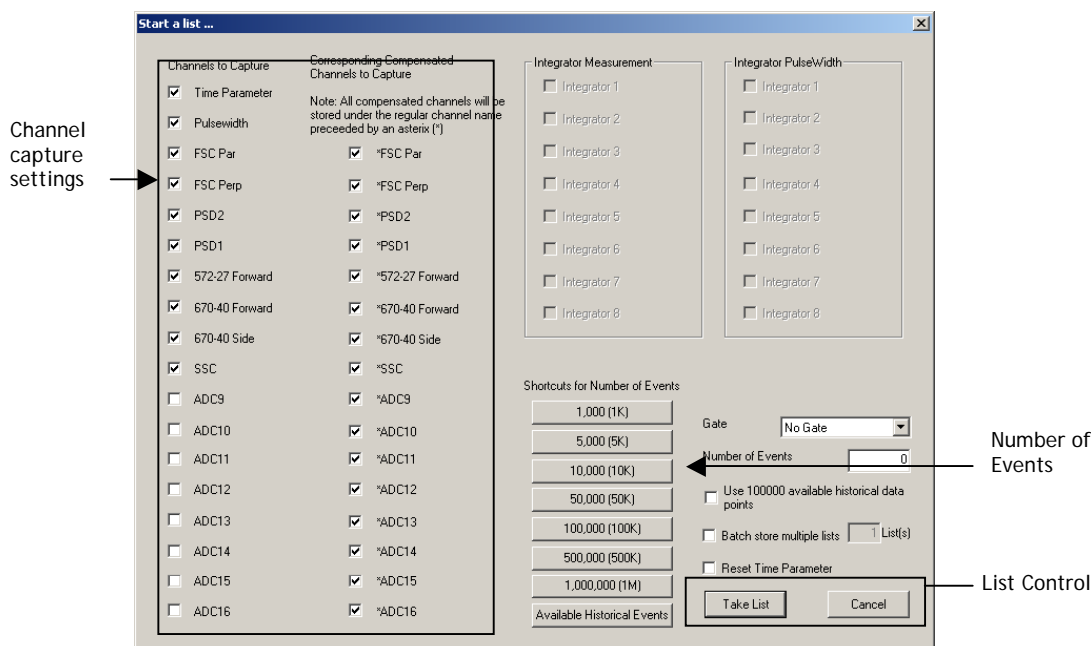
Figure 4.14: List Tools

Channel Capture Settings

Spigot has the ability to capture data and store it in files using the standard flow cytometry format (*.fcs).

This data capture is configured using the List Tool, available by clicking _LIST_ (or by selecting **GLOBAL ACTIONS⇒TAKE A LIST** from the drop down list on the menu bar).

In the _Start a List_ dialog box that appears (shown below) specific channels and parameters may be checked for inclusion in the data capture.

Number of Event Selection

The number of events to be captured may be set in the center of the dialog box, either by typing numbers in the window or by clicking on one of the shortcut buttons placed under the window for user convenience.

Miscellaneous Settings

If the user wishes to store data that only falls through a gate, ie selected for left or right sort, the gate tool can be used to do so.

Note that all other data will not be stored.

Historical data may be available to be stored in a list. This means that even if data is not running, Spigot's built in buffer may have previous data available for storage.

Check in the box Use Historical Data if you wish to use this feature.

Lists may be batch stored by checking the _Batch Store Multiple Lists_ box. The number of lists to batch store may also be selected.

The time parameter may be reset when a list is taken by checking the box _Reset Time Parameter_.

List Control

After setting these list parameters, clicking _Take List_ starts the data capture. A _List Options_ dialog box (shown below) also appears for attaching reference information to the file being created.

Clicking on _Save List_ brings up a standard Windows "Save As" dialog box for naming and saving the new list in a preferred location.

Values entered into the List Options dialog will be saved in the *.fcs file header and can be recalled in an analysis package.

Figure 4.15: List Options Dialog

5

Maintenance

Summary

This chapter covers recommended maintenance of the instrument and provides operators with contact information for customer support at BD if technical assistance is needed.

Periodic User Cleaning and Inspection

Daily Maintenance

After each day's operation, the fluidics system should be cleared and dried out thoroughly using the following procedure:

1. Turn air switch to off

2. Release sheath reservoir pressure, then disconnect, drain and rinse reservoir.

3. Refill sheath reservoir with 100 mL of de-ionized water, reconnect sheath reservoir and turn air switch on.

4. Run this fluid through entire system, including backflushing the sample line, until the reservoir runs dry.

5. Continue to run air through system for approximately 15 minutes, ensuring that all lines are free of fluids.

6. During this time, the nozzle tip may be removed, sonicated if desired, and left to dry in a protected area.

7. Shut down fluidics by turning off air switch and vacuum pump.

8. Empty, rinse and air dry both reservoirs.

Weekly Maintenance

To keep the instrument running well for a long time, perform the following on at least weekly basis:

1. Remove dust from all exposed surfaces.

2. Clean salt buildups from the parts and areas exposed to sheath or sample fluids.

3. Clean the WDU tray.

4. Vacuum dust and lint from fan areas, such as the back of the sort electronics console, laser power supplies, and the computer.

5. Inspect tubing and fluidics for leaks.

Operator Maintenance

Fluidics

The instrument has been designed so that all fluidics lines are replaceable by the user.

To order replacement assemblies, and for guidance on installing these parts, please contact BD Customer Service.

	Warning
⚠️	**The instrument has been tested and validated using specific fluidics tubing. The use of any tubing other than that provided by or specified by BD may result in substandard instrument performance, damaging leakage or instrument failure, and will not be supported by BD.**

Optics

The instrument has been designed so that filters in the detector modules may be removed or interchanged by the user.

Please contact BD Customer Service for guidance on this process and to order additional or replacement filters.

Other Maintenance

Any other maintenance should not be considered routine and should only be performed by BD service technicians.

Please contact BD for service arrangements using the contact information below.

A

Drop Delay Worksheet

PSI: _____   B.O.P:_____

Clock:_____   Piezo: _____

Delay Setting:_____

Delay Setting:_____

Delay Setting:_____

Delay Setting:_____

Delay Setting:_____

Delay Setting:_____

Delay Setting:_____

Delay Setting:_____

Delay Setting:_____

Delay Setting:_____

Instrument: _____ Operator: _____ Date: _____

B

Pressure / Frequency Chart

Use the following chart as a guideline for approximate available Pressure / Frequency settings. Actual frequency settings may be slightly different for each nozzle assembly. Scan the frequencies near the recommended values to find the frequency with the shortest break off point.

Tip Size	Pressure (PSI)	Drop Rate (HZ)
50	10	53750
50	13	61750
50	14.5	68000
50	17	73750
50	18.5	78813
50	22	87813
50	25	93750
50	27.5	101125
50	33	111750
50	41	126250
50	44	130813
50	47	137500
50	59	157063
50	72	173438
50	76	180063
50	85	192500
70	7	28688
70	12.3	39250
70	18.5	49188
70	22	53750
70	27.5	61750
70	33	68000
70	39.3	74188
70	45	78813

70	52	87813
100	5.2	15000
100	8.5	19875
100	10.2	22625
100	15	27313
150	4.5	10000

C

Available Lasers, 2007

The flow cytometer is equipped with one or more of the following embedded lasers. All embedded lasers are certified by their respective manufacturers to comply with the requirements set out in the relevant international standards (IEC 60825-1, 21 CFR 1040.10.)

The original manufacturer's documentation has been provided with each laser

Wavelength	Power	Manufacturer	Product	Mount
488nm	100, 200mW	Coherent	Sapphire 488	Rear
355nm	20, 60, 100mW	JDS Uniphase	XCyte	Rear
405nm	50mW	Coherent	Cube	Side
635nm	30mW	Coherent	Cube	Side
532nm	50, 100, 150 mW	Cobolt	Samba	Side
561nm	50, 75 mW	Cobolt	Jive	Side

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